Plasticité phénotypique de la résistance à la dessiccation chez les moustiques Anopheles coluzzii et An. gambiae en Afrique sub-saharienne

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Résumé

Dans les savanes d'Afrique de l'Ouest, les moustiques *Anopheles coluzzii* et *An. gambiae,* qui transmettent le paludisme, pullulent en saison des pluies et disparaissent en saison sèche. Les mécanismes permettant le maintien des populations d'Anophèles d'une année sur l'autre restent inconnus et difficiles à mettre en évidence sur le terrain. Au cours de ce travail, nous avons étudié, par des approches comparatives et expérimentales, la plasticité physiologique et morphologique des femelles *An. coluzzii* et *An. gambiae* exprimée en réponse aux conditions déshydratantes de la saison sèche. Des analyses métabolomiques, protéomiques, morphométriques et respirométriques, nous ont permis de mettre en évidence une grande variabilité des profils exprimés entre les espèces et entre les différentes populations d'*An. coluzzii*. Alors que nos résultats suggèrent une capacité de dispersion accrue chez les jeunes adultes d'*An. gambiae* en saison sèche, l'accumulation de métabolites à fonction osmoprotectrice et/ou impliqués dans la perméabilité de la cuticule semblent renforcer la tolérance à la dessiccation chez *An. coluzzii*. Ces données permettent de mieux comprendre la dynamique des populations d'Anophèles en Afrique. Elles ouvrent également de nouvelles perspectives de recherche sur les stratégies de survie de ces insectes en environnement défavorable.

Mots clés : dessiccation; *Anopheles gambiae s.l.*; plasticité phénotypique; estivation; Burkina-Faso

Abstract

In dry savannahs of West Africa, the malarial mosquitoes *Anopheles coluzzii* and *An. gambiae* swarm during the rainy season but almost disappear at the onset of the dry season. Interestingly, the ecological and physiological bases enhancing the survival of these insects during the desiccating conditions of the dry season remain unknown, and hard to understand with field studies. In the present work, we conducted experimental and comparative studies to analyse the physiological and morphological plasticity of female *An. coluzzii* and *An. gambiae* mosquitoes in response to the environmental conditions of the dry season. Metabolomic, proteomic, morphometric and gas exchange analyses highlighted a wide range of responses to the dry season conditions in these species, and enables distinguishing the two considered species. Differences were also observed between populations of the *An. coluzzii* mosquitoes from distinct habitats. The young females of *An. gambiae* seem characterised by higher dispersal abilities. In *An. coluzzii* females, the increasing amounts of osmoprotectants and metabolites involved in cuticle permeability suggest increased desiccation resistance at the onset of the dry season. These new results contribute improving our understanding of the seasonal population dynamics of anopheline mosquitoes in West Africa. The present work opens new research perspectives in our understanding of the survival strategies of these malarial vectors during the unsuitable environmental conditions of the dry season.

Key words: desiccation; *Anopheles gambiae s.l.*; phenotypic plasticity; aestivation; Burkina-Faso

"It is not the stronger of the species, nor the most intelligent that survives. It is the one that is the most adaptable to change"

Charles Darwin

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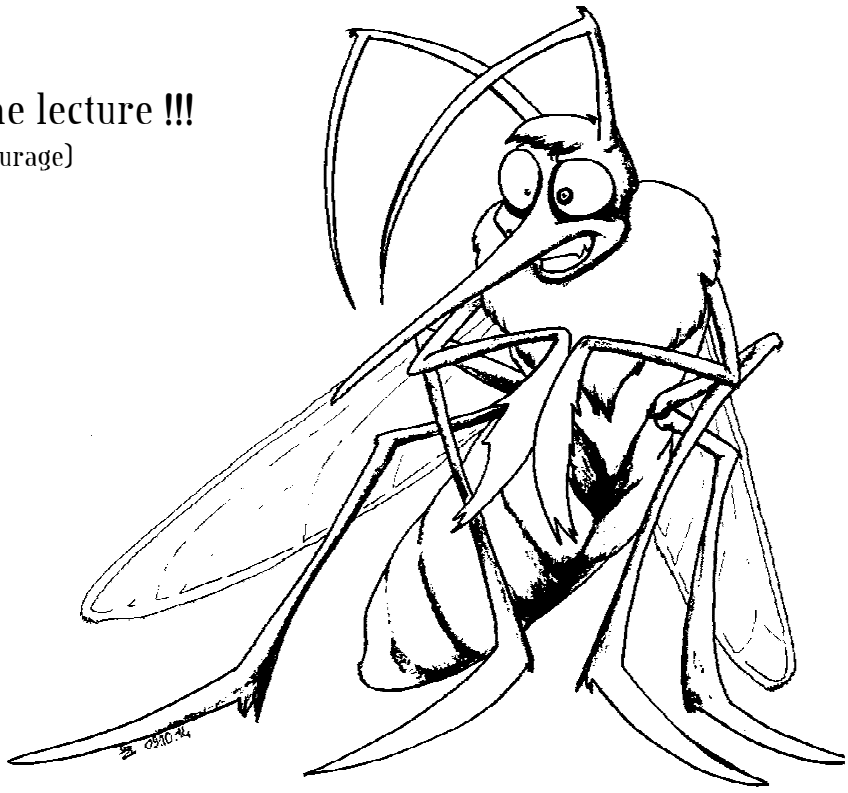
MERCI & Bonne lecture !!!
(si vous en avez le courage)



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1. L'environnement et ses contraintes

1.1. L'environnement: un système hétérogène et dynamique

La distribution, l'écologie et l'évolution des espèces sont influencées par les rapports qu'entretiennent les organismes avec leurs environnements biotiques et abiotiques (Addo-Bediako *et al.*, 2000 ; Pickett & White, 1985 ; Sorensen & Loeschcke, 2007). Par définition, l'environnement abiotique se réfère à l'ensemble des paramètres physico-chimiques des écosystèmes tels que la température, l'humidité relative, la disponibilité des ressources trophiques et de l'eau, la salinité, les rayonnements ultraviolets, etc. Ces paramètres peuvent varier conjointement ou de manière découplée, et, quelques soient leurs types de variation, peuvent avoir des effets synergiques, positifs ou négatifs sur la biologie des organismes. Parallèlement à l'environnement abiotique s'ajoutent des pressions d'ordre biotique qui recensent l'ensemble des interactions entre les entités biologiques en présence. Ces interactions incluent par exemple la prédation, la compétition ou bien encore le parasitisme.

Les paramètres environnementaux abiotiques et biotiques fluctuent à la fois dans l'espace et dans le temps. Dans l'espace, l'environnement est considéré comme hétérogène lorsque sa variabilité est inférieure à la capacité de dispersion naturelle de l'espèce considérée (Levins, 1968). En revanche, l'environnement est considéré comme spatialement uniforme lorsqu'il varie au-delà de la disperssion de l'espèce considérée. L'environnement est également hétérogène sur le plan temporel et peut varier sur des échelles de temps de l'ordre de la minute, de la journée, du mois ou encore de la saison.

1.2. Environnements hétérogènes et plasticité phénotypique

La plasticité phénotypique est reconnue comme un moyen mis en place par les organismes pour maximiser leur valeur adaptative[1] dans un environnement changeant (Chown *et al.*, 2007 ; Davidson *et al.*, 2011 ; Richards *et al.*, 2006). Définir la plasticité phénotypique a longtemps été un sujet de controverses. La première définition de la plasticité phénotypique remonte à Bradshaw (1965). Cet auteur définit la plasticité comme la variabilité d'expression des traits d'un individu dans différents environnements. West-Eberhard (2003) a ensuite repris cette définition : « la capacité

[1] Capacité d'un individu à transmettre ses gènes à la génération suivante (Hamilton, 1964). Egalement nommé valeur adaptative ou succès reproducteur.

d'un organisme à réagir à une variable environnementale par un changement de forme, d'état, de mouvement ou encore de taux d'activité ». Puis, Ananthakrishnan & Whitman (2005) ont défini la plasticité comme « l'expression de différents phénotypes à partir d'un même génotype ». Nous retiendrons cette dernière définition, à laquelle Freeman (2007) ajoutent que « l'expression de différents phénotypes à partir d'un même génotype est induite par un environnement changeant ».

Initialement, la plasticité phénotypique a été utilisée pour décrire les changements morphologiques associés aux conditions environnementales pendant le développement des organismes (Pigliucci, 1996). Aujourd'hui, cette notion comprend l'ensemble des réponses comportementales, morphologiques, physiologiques et biologiques permanentes ou temporaires induites par la variabilité de l'environnement. L'outil de recherche de la plasticité phénotypique d'un organisme est la norme de réaction qui défini la relation entre les environnements auxquels les organismes d'un génotype donné peuvent être confrontés et les différents phénotypes produits par ce génotype (Pigliucci, 2001). Les normes de réactions peuvent être décrites par des modèles de régressions polynomiaux ou linéaires. Dans le cas des modèles de régression linéaire, les normes de réactions sont simplement décrites par une droite caractérisée par sa pente et son ordonnée à l'origine. La **Fig. 1** représente graphiquement les trois régressions linéaires théoriques définis par les normes de réaction : la variation génétique **(Fig. 1a**), la plasticité phénotypique (**Fig. 1b**) et la variation génétique de la plasticité phénotypique (**Fig. 1c**).

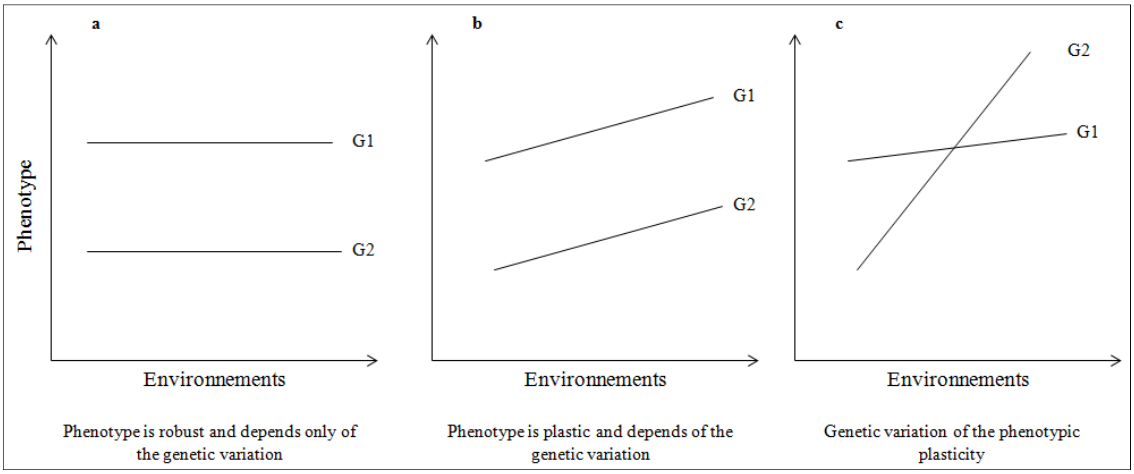


Fig. 1 Représentation graphique du concept de variation génétique (a), plasticité phénotypique (b) et variation génétique de la plasticité phénotypique (c). D'après Pigliucci (2001).

D'après Pigliucci (2001), les différentes composantes de la norme de réaction peuvent être estimées par une analyse des variances :
$V_P=V_G+V_E+V_{GxE}+V_{err}$, où :

- V_P représente la variance phénotypique totale ;
- V_G représente la variance phénotypique attribuée aux différences entre génotypes. Ce terme est significatif même en absence de plasticité phénotypique (norme de réaction horizontale ; **Fig. 1a**) et/ou de variation génétique de la plasticité phénotypique (normes de réactions parallèles ; **Fig. 1b**) ;
- V_E représente la variance phénotypique attribuée aux changements environnementaux. Ce terme est significatif si les génotypes expriment des phénotypes différents selon l'environnement (**Fig. 1c**), et même si ces génotypes réagissent de la même façon (absence de variation génétique de la plasticité phénotypique ; **Fig. 1b**) ;
- V_{GxE} représente l'interaction des différences entre les génotypes et les changements environnementaux. Ce terme est significatif lorsque les différents génotypes ne répondent pas de la même façon aux différents environnements (variation génétique de la plasticité phénotypique ; **Fig. 1c**) ;
- V_{err} représente la variance résiduelle incluant les erreurs expérimentales.

1.3. La plasticité phénotypique est renforcée par le stress

Il arrive parfois que les variations spatiales et/ou temporelles des paramètres environnementaux représentent un état de gêne pour les espèces. Cet état de gêne, nommé contrainte en biologie des organismes, correspond à une modification une diminution de la performance pour un trait donné, induite par un changement de l'environnement. En fonction de l'intensité, de la fréquence et de la durée des variations des paramètres environnementaux, la contrainte peut s'intensifier et entrainer un état de stress (Denlinger & Armbruster, 2014). En effet, pour une variable environnementale donnée, les organismes possèdent un optimum pour lequel la performance d'un trait ou d'un ensemble de traits donné est optimale. En revanche, en s'éloignant de cet optimum, les performances des organismes diminuent, il s'agit de l'état de contrainte. En s'éloignant encore plus loin de part et d'autre de l'optimum, les performances s'amoindrissent encore, et l'insecte entre dans un état de stress (**Fig. 2**).

Du niveau cellulaire au niveau individuel, le stress se traduit par une perturbation de l'homéostasie[1] au sein des organismes et par une diminution de leur performance et de leur valeur adaptative (Hoffmann & Parsons, 1991 ; Kültz, 2005 ; Sorensen & Loeschcke, 2007).

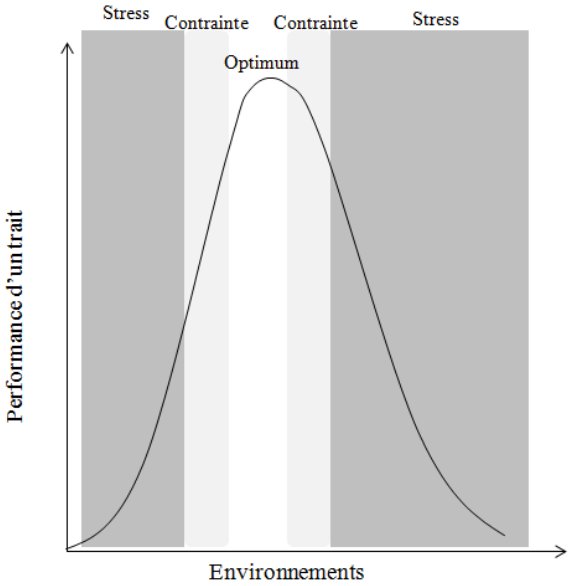


Fig.2 Construction schématique de la notion de contrainte et de stress pour un trait donné en fonction de l'environnement. D'après Jensen (1906).

Il existe de nombreux exemples dans la littérature témoignant de l'effet bénéfique de la plasticité phénotypique sur les espèces exposées à des changements contraignants et/ou stressants de leur environnement. Il est, en effet, communément admis que la plasticité phénotypique permet de minimiser le degré de stress subit par les organismes présents dans un environnement changeant (Article V, VII ; Foray *et al.*, 2013 ; Miner *et al.*, 2005 ; Terblanche & Chown, 2006).

1.4. La dessiccation: un facteur de stress pour les insectes terrestres

Chez les organismes terrestres, et en particulier les insectes, la dessiccation des tissus corporels se fait essentiellement par transpiration passive au travers de la cuticule, puis les échanges gazeux avec l'environnement et enfin les excrétions orales et anales (Chown, 2002 ; Hadley, 1994 ; Quinlan & Gibbs, 2006). La dessiccation est un phénomène naturel chez les organismes terrestres, cependant dans certaines conditions, les variations conjointes de la température et de l'humidité relative augmentent les processus de dessiccation et induisent un état de stress chez les organismes (Addo-Bediako *et al.*, 2000; Chown, 1993; Chown & Nicholson, 2004 ; **Fig. 3**). Le risque de

[1] Capacité d'un système à conserver un état d'équilibre en dépit des contraintes extérieures (Bernard, 2011).

dessiccation peut également être lié pour certains organismes à une élévation de la salinité du milieu (Article V, VII ; White *et al.*, 2013) ou encore le gel des sources d'eau pendant les périodes hivernales. Dans ce dernier cas, nous parlons de cryodessiccation. Dans de tel environnement, compenser les pertes d'eau en assimilant l'eau n'est pas suffisant et les organismes doivent développer des mécanismes de réponses physiologiques, morphologiques et/ou comportementales afin de résister à la dessiccation.

La dessiccation des tissus corporels des organismes peut être à l'origine d'un état de stress majeur chez les insectes terrestres lorsque la quantité d'eau perdue dépasse le seuil de tolérance des individus. D'après Schmidt-Nielsen (1997) *«The greatest physiological advantage of terrestrial life is the easy access to oxygen; the greatest physiological threat to life on land is the danger of dehydration »*. En effet, la dessiccation peut induire de nombreuses perturbations, incluant une diminution de l'activité locomotrice (Hoffmann & Parsons, 1993), de la reproduction (Benoit *et al.*, 2010a) ou encore de la longévité des individus (Hoffmann & Harshman, 1999). Au niveau cellulaire, la dessiccation altère (1) l'homéostasie de la balance hydrique et osmotique entre les compartiments inter- et intra-cellulaires des organismes, (2) la fluidité des membranes lipidiques, (3) les protéines et enzymes (dénaturation et agglomération liées à une perturbation de leurs propriétés physico-chimiques et des liaisons hydrogènes qui maintiennent les structures) et (4) l'accumulation de dérivés réactifs de l'oxygène ou *Reactive Oxygen Species* (ROS ; ex. ozone, péroxyde d'oxygène, etc.) capables d'oxyder et de détruire les protéines, l'ADN et les membranes des cellules (**Fig.3**).

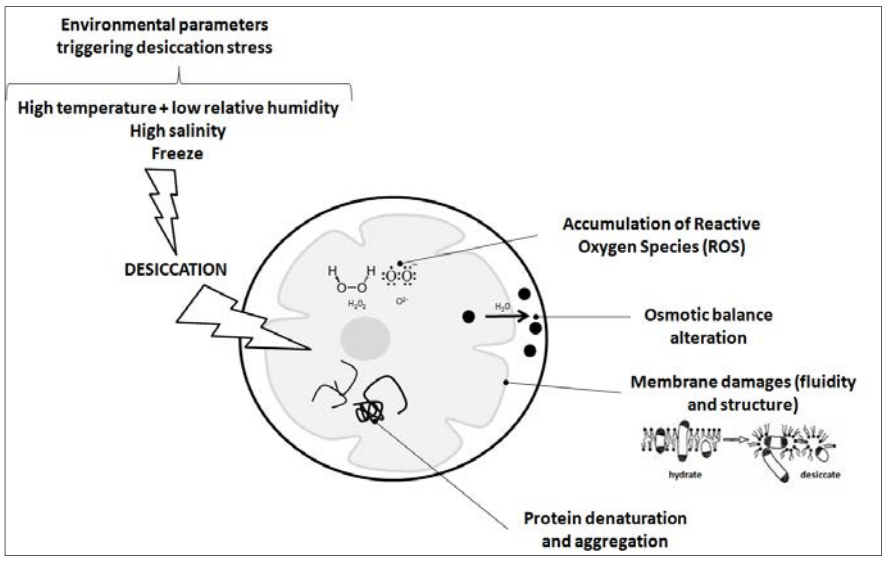


Fig. 3 Construction schématique des principaux dommages cellulaires causés par la dessiccation chez les insectes terrestres.

2. Principaux mécanismes de réponse des insectes à la dessiccation

« … work has been concerned with the exploration of particular mechanisms, and there is now both a need and an opportunity to take a wider view and attempt to synthesise such knowledge into an understanding of water balance in whole animals in natural situations… » Edney (1977)

2.1. Ajustement de la composition biochimique de la cuticule

La transpiration passive des insectes à travers la cuticule constitue la principale voie de dessiccation chez les insectes terrestres (Chown, 2002 ; Chown & Nicholson, 2004 ; Hadley, 1994 ; Johnson & Gibbs, 2004). Par exemple, chez les femelles *Pogonomyrmex barbatus* (Hymenoptera, Formicidae), les pertes d'eau associées à la transpiration cuticulaire représentent l'essentiel des pertes totales enregistrées (Johnson & Gibbs, 2004 ; **Fig. 4**). De nombreuses études ont examiné comment les insectes terrestres, et en particulier les espèces résidant au sein d'habitats arides, limitent la transpiration cuticulaire. De façon générale, les espèces résidant au sein d'habitats arides présentent une transpiration cuticulaire significativement réduite comparée à leurs congénères des régions plus humides (Edney, 1977 ; Gibbs *et al.*, 1998 ; Hadley, 1994). Cette différence est en grande partie due aux propriétés hydrophobes de la cuticule.

Le caractère hydrophobe de la cuticule est principalement assuré par sa composition lipidique (Hadley, 1994). En effet, il a été démontré qu'une modification de la composition quantitative et qualitative des lipides constituant la couche la plus externe de la cuticule (l'épicuticule) altère significativement la transpiration cuticulaire des insectes (Gibbs & Rajpurohit, 2010; Nelson & Lee, 2004 ; Wagoner *et al.*, 2014). De façon générale, le caractère hydrophobe des lipides repose sur le principe de fusion ou de transition critique de la température (CTT ; Benoit *et al.*, 2005 ; Hadley, 1994). Succinctement, ce principe est basé sur la température (température critique ; T_c) à laquelle les composés lipidiques passent d'un état solide imperméable à un état fluide perméable. La capacité hydrophobe d'un composé lipidique correspond donc à la valeur de sa T_c. Ainsi, dans un contexte de dessiccation par élévation des températures (ex. saison sèche en Afrique), plus la T_c est élevée, plus la chaine lipidique est considérée comme hydrophobe. Parmi les lipides conférant un haut niveau d'hydrophobicité à la cuticule, les hydrocarbones à longues chaines carbonées et leurs dérivés sont les plus documentés dans la littérature (Gibbs *et al.*, 1998). Certaines espèces, tels que les moustiques *Anopheles*

gambiae (Diptera, Culicidae), montrent ainsi une augmentation quantitative de la composition en hydrocarbones à longues chaines (n-alkanes) au niveau de l'épicuticule à l'approche de la saison sèche (Wagoner *et al.*, 2014). Une autre étude chez le papillon *Antheraea yamamai* (Lepidoptera, Saturnidae) montre une sur-expression de l'enzyme CYP4G, responsable de la synthèse d'hydrocarbones cuticulaires, quelques temps avant l'établissement de conditions environnementales déshydratantes (Yang *et al.*, 2008).

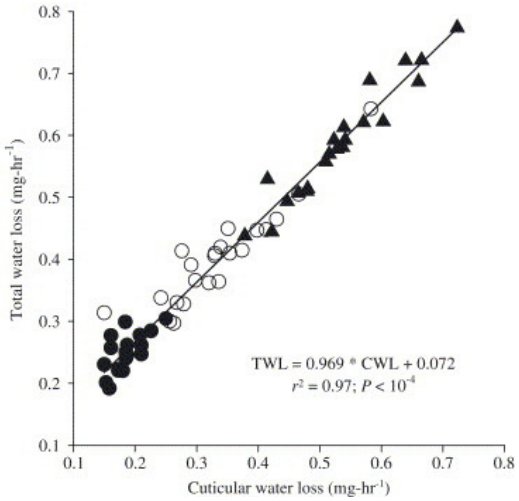


Fig. 4 Corrélation positive entre les pertes en eau au travers la cuticule et la quantité totale d'eau perdue par les femelles *Pogonomyrmex barbatus* (Hymenoptera, Formicidae). Les différents symboles indiquent des différences au niveau du statut d'accouplement. Les cercles pleins représentent les femelles non accouplées ; les cercles ouverts représentent les femelles accouplées ; les triangles pleins représentent les femelles après construction du gîte de pontes ; les triangles ouverts représentent les femelles avant construction du gîte de ponte. D'après Johnson & Gibbs (2004).

La cuticule des insectes terrestres est également constituée de sucres, de protéines et de peptides riches en acides aminés. L'ajustement quantitatif et qualitatif de ces composés permet également de modifier la perméabilité de la cuticule. L'accumulation d'acides aminés aromatiques tels que la phénylalanine, le tryptophane ou encore la tyrosine peut notamment influencer le degré d'hydrophobicité de la cuticule (Andersen, 1979). D'ailleurs, certaines espèces d'insectes tendent à favoriser une alimentation riche en ces acides aminés lorsqu'elles sont exposées à des conditions déshydratantes (Behmer & Joern, 1993). Ces composés sont, en effet, connus pour favoriser les processus de sclérotisation de la cuticule. D'après certaines études, une augmentation du degré de sclérotisation de l'endocuticule (sous-couche sclérotisée de la procuticule) participerait à limiter les pertes en eau des organismes (Hadley, 1994 ; Machin & Lampert, 1987). En effet, la sclérotine, une protéine tannée et hydrophobe, est connue pour accroitre la densité, la rigidité et l'imperméabilité des tissus dans laquelle elle s'exprime (Benoit *et al.*, 2005). D'autres protéines cuticulaires, telles que les « *RR-2 cuticular proteins* », semblent également

impliquées dans le caractère hydrophobe des cuticules (Benoit *et al.*, 2010a ; Karouzou *et al.*, 2007). Cependant, l'implication directe de telles protéines dans la résistance à la dessiccation des insectes terrestres n'a pas été encore démontrée.

2.2. Minimisation des pertes d'eau associées aux échanges gazeux

Outre la transpiration cuticulaire, les échanges gazeux des organismes avec leurs environnements constituent la seconde source de dessiccation chez les insectes terrestres. En effet, ces pertes représentent en moyenne entre 5 et 20 % de la totalité du volume d'eau perdue par les organismes et ont lieu pendant l'ouverture des spiracles (Chown, 2002 ; Hadley, 1994 ; **Fig. 5**). Les pertes d'eau pendant les échanges gazeux sont d'autant plus importantes que l'environnement est aride et sec. Les organismes résidant dans ce type d'environnement ont donc dû développer des mécanismes particuliers afin de limiter ce type de pertes en eau.

Certaines espèces d'insectes, tel que le ténébrion *Eleodes armata* (Coleoptera, Tenebrionidae) ou encore le scarabée araignée *Mezium affine* (Coleoptera, Anobiidae), ont ainsi développé des structures morphologiques permettant d'accroitre localement l'humidité relative autour des spiracles, et ainsi réduire la dessiccation. Ce résultat peut être obtenu par la création d'une cavité interne (ex. soudure des élytres) créant un milieu confiné et à humidité élevée autour des spiracles, ou par une réduction de la proximité physique entre les spiracles (Benoit *et al.*, 2005 ; Cloudsey-Thompson, 2001). Chez d'autres espèces, des structures lipidiques hydrophobes (ex. trichomes) sont synthétisées en bordure des spiracles afin de limiter la dessiccation des insectes au cours des échanges gazeux. Ce type de structure a été récemment mis en évidence à l'approche de la saison sèche chez les femelles moustiques du genre *Anopheles* (Diptera, Culicidae), alors qu'elles sont absentes pendant la saison des pluies chez ces mêmes femelles (Mamai *et al.*, in revision for Parasites and vectors; **Fig. 6**).

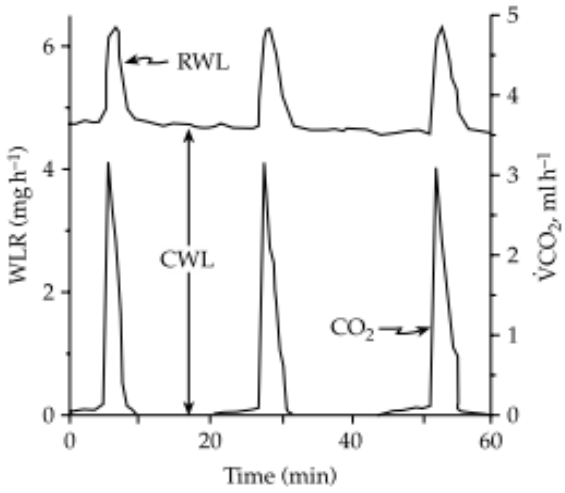


Fig. 5 Enregistrement simultané des pertes en eau (WLR en mg/h ; graphique supérieur) et des émissions en CO_2 (VCO_2 en ml/h ; graphique inférieur) chez *Taeniopoda eques* (Orthoptera, Romaleidae) à 25 °C (Quinlan & Hadley, 1993).

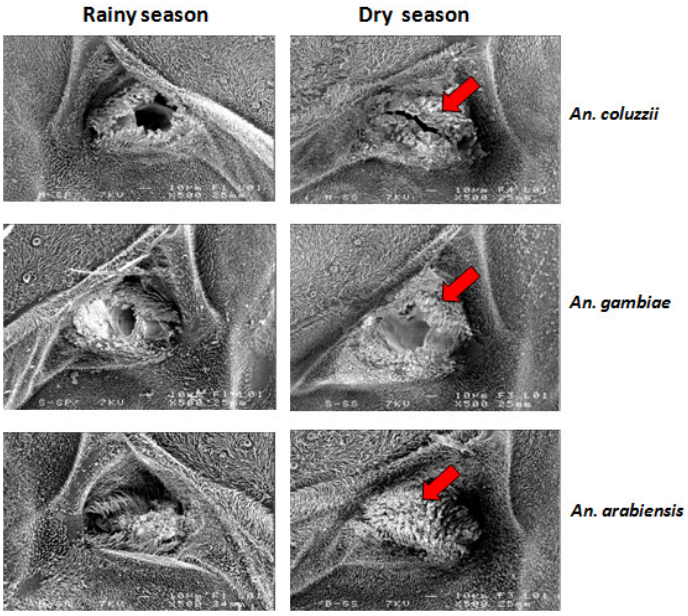


Fig. 6 Ajustements morphologiques des spiracles mésothoraciques en saison sèche et saison des pluies chez les espèces du complexe *Anopheles gambiae s.l.* au Burkina-Faso. Au delà de l'aspect ouvert ou fermé des spiracles, des structures (trichomes) hydrophobes bordent l'ensemble des spiracles en saison sèche. Les flèches rouges illustrent la localisation de ces structures (Mamai *et al.*, in revision for Parasites and vectors).

Une autre stratégie consiste à réduire partiellement ou totalement les échanges gazeux et donc l'activité métabolique des insectes (Chown, 2002 ; Davis *et al.*, 2000 ; Hadley, 1994 ; Rourke, 2000). La relation entre le maintien de la balance hydrique et la réduction du taux métabolique a été relativement bien étudiée chez les insectes à l'état de dormance[1] (Denlinger, 2002 ; Storey & Storey, 1990; Tanaka *et al.*, 1988). Chez ces organismes, le temps et la fréquence d'ouverture des spiracles sont réduits au minimum nécessaire pour assurer les fonctions biologiques des individus, limitant ainsi les pertes en eau corporelle. Une activité discontinue des échanges gazeux peut également permettre de limiter la déshydratation des organismes. Cependant, le réel

[1] Ensemble des états physiologiques induisant un arrêt des processus biologiques et une suppression de l'activité métabolique. Parmi les états physiologiques de la dormance, on retrouve la quiescence et la diapause (Koštál , 2006).

bénéfice des processus de réduction des échanges gazeux sur la minimisation des pertes en eau au sein des organismes est à ce jour controversé (Chown, 2002). De nombreuses études montrent, en effet, que la contribution des échanges gazeux dans la dessiccation des insectes doit être minimisée par rapport à celle induite par la transpiration cuticulaire (Article II ; Bradley *et al.*, 1999; Djawdan *et al.*, 1997 ; Rourke, 2000 ; Williams *et al.*, 1998 ; Williams & Bradley, 1998 ; **Tableau 1**). D'après ces auteurs, la réduction de l'activité de respiration chez les insectes ne constituerait donc pas un réel bénéfice pour l'ajustement de la balance hydrique (Chown, 2002). La réduction des échanges gazeux serait alors la résultante d'une réduction de l'activité métabolique liée à la mise en place d'une stratégie de dormance pendant laquelle les insectes résistent mieux à la dessiccation. A ce jour, des études plus poussées sont nécessaires afin d'élucider ce point de discorde dans la littérature.

Tableau 1. Contribution relative (%) de la respiration et de la transpiration cuticulaire aux pertes en eau totales chez différentes espèces d'insectes terrestres. Résultats issus des travaux de Chown (2002).

Species	Respiratory	Cuticular	Temperature	Reference
Blattodea				
Periplaneta Americana	13	87	20	Machin *et al.*, 1991
Orthoptera				
Melanoplus sanguinipes	15	85	25	Rourke, 2000
Romalea guttata	3	97	25	Quinlan & Hadley, 1993
Taeniopoda eques	4.8	95.2	25	Quinlan & Hadley, 1993
Coleoptera				
Aphodius fossor	5	97	15	Chown & Holter, 2000
Eleodes armata	3	97	25	Ahearn, 1970
Omorgus radula	6.5	93.5	24	Bosch *et al.*, 2000
Hymenoptera				
Camponotus vicinus	2	98	25	Lighton, 1992
Cataglyphis bicolor	8	92	25	Lighton, 1992
Pogonomyrmex occidentalis	3.5	96.5	25	Quinlan & Lighton, 1999
Pogonomyrmex rugosus	13	87	25	Lighton *et al.*, 1993
Pogonomyrmex rugosus	2.4	97.6	25	Quinlan & Lighton, 1999

2.3. Réabsorption de l'eau au niveau du rectum et du système cryptonéphridien

Le système d'excrétion des déchets métaboliques constitue la troisième voie de dessiccation chez les insectes après la transpiration cuticulaire et les échanges gazeux. Des mécanismes de régulation des pertes en eau au niveau de ce système sont donc

nécessaires au maintien de l'homéostasie des individus. Il a ainsi été démontré que la quantité d'eau excrétée par les espèces dépend des conditions environnementales auxquelles elles sont exposées (Gilles, 2006). Par exemple, la quantité d'eau contenue dans la matière fécale produite par la mouche tsé-tsé *Glossina pallides* (Diptera, Glossinidae) dépend des conditions d'humidité relative dans lesquelles se trouve l'insecte (Bursell, 1960). Ainsi, dans les milieux où l'humidité relative est élevée la matière fécale est constituée à 75% d'eau, tandis que dans les milieux arides elle ne contient plus que 35% d'eau.

Les processus de régulation du système d'excrétion de l'eau sont localisés au niveau du rectum et dans la région proximale des tubes de Malpighi (Chown & Nicholson, 2004). Ces processus utilisent le principe d'osmose de l'eau par le transport de molécules inorganiques (Na^+, K^+, Cl^-, etc.), à travers des pompes énergie-dépendantes. Parmi ces pompes, il a été mis en évidence qu'une sur-expression de la pompe Na^+/K^+-ATPase, au niveau du rectum, était à l'origine d'une meilleure réabsorption de l'eau et d'un meilleur taux de survie des individus en conditions déshydratantes chez *Drosophila melanogaster* (Diptera, Drosophilidae) (Folk & Bradley, 2004). D'autres études conduites chez le moustique *Anopheles merus* (Diptera, Culicidae) montrent que la localisation et l'abondance de ces pompes varient selon que les moustiques soient ou non exposés à des conditions déshydratantes (White *et al.*, 2013 ; Smith *et al.*, 2008; 2010). D'après des analyses immunochimiques, il a ainsi été mis en évidence que ces pompes sont principalement localisées au niveau de la région antérieure/dorsale du rectum où les processus de réabsorption de l'eau sont les plus intenses (White *et al.*, 2013 ; **Fig. 7**). Il est par ailleurs démontré que l'activité des pompes Na^+/K^+-ATPase est régulée par des hormones antidiurétiques (Coast *et al.*, 2002 ; Gäde, 2004 ; Riehle *et al.*, 2002). Parmi ces hormones, les peptides Capa régulent les voies signalétiques du calcium (Ca^{2+}) et de la guanosine monophosphate (cGMP) qui modulent la diurèse chez les insectes terrestres (Davies *et al.*, 2013). L'expression de ce peptide réduit significativement la production d'urine et leurs récepteurs ont été observés chez la Drosophile (Park *et al.*, 2002; Terhzaz *et al.*, 2012), chez *An. gambiae* (Olsen *et al.*, 2007; Pollock *et al.*, 2004) et chez *Rhodnius prolixus* (Hemiptera, Reduviidae) (Paluzzi *et al.*, 2010). Cependant,

la relation existant entre l'expression de ces peptides et la capacité de résistance à la dessiccation des insectes reste peu documentée.

D'autre part, chez certaines espèces d'insectes, les tubes de Malpighi peuvent adopter une conformation particulière facilitant la réabsorption de l'eau. Cette conformation particulière est nommée système cryptonéphridien, et correspond à un rapprochement physique de la région distale des tubes de Malpighi avec le rectum (**Fig. 8**). Une membrane périnéphritique englobe le système afin de créer un système clos entre la zone d'absorption et de sécrétion de l'eau dans l'hémolymphe. Ce réarrangement particulier du système d'excrétion est particulièrement développé par des espèces habitant les régions sèches et arides telles les larves de *Tenebrio molitor* (Coleoptera, Tenebrionidae), mais également certaines espèces d'Hyménoptères, de Neuroptères et de Lépidoptères (Grimstone *et al.*, 1968 ; Ramsay, 1964 ; Saini, 1964).

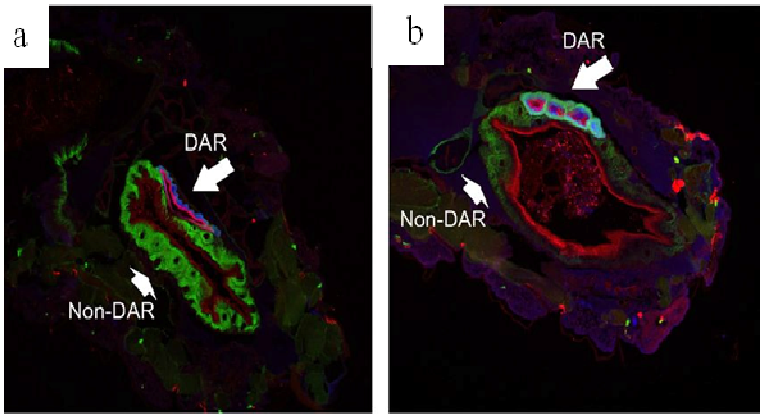


Fig. 7 Localisation des pompes Na^+/K^+-ATPase (vert) et des anhydrases carbonique (bleu cyan) indicatrices de la présence des cellules de la région antérieure/dorsale du rectum (DAR) chez *Anopheles merus* exposé à des conditions non déshydratantes (eau douce, a) ou déshydratantes (eau salée, b). Les résultats montrent une relocalisation des pompes Na^+/K^+-ATPase au niveau des cellules de la région antérieure/dorsale du rectum chez les individus exposés aux conditions déshydratantes (b). D'après White *et al.* (2013).

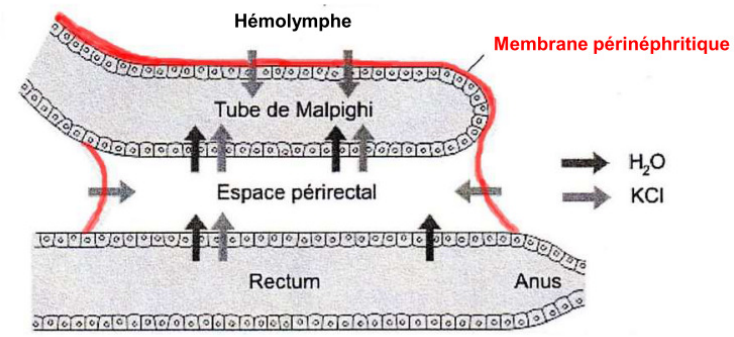


Fig. 8 Illustration schématique du système cryptonéphridien chez *Tenebrio molitor*.

2.4. Synthèse de protéines

2.4.1. Protéines chaperonnes

Les protéines chaperonnes jouent un rôle primordial dans le maintien de l'homéostasie du protéome intracellulaire. Ces protéines agissent seules où en synergie avec d'autres protéines afin de réparer et/ou d'éliminer les protéines dénaturées et agrégées, produites par la dessiccation des tissus corporels (Feder & Hofmann, 1999 ; **Fig. 9**). Parmi ces protéines, la famille des protéines de choc thermique ou *Heat-shock Proteins* (Hsp) sont les mieux décrites dans la littérature entomologique (Benoit *et al.*, 2010b ; Feder & Hofmann, 1999; Parsell & Lindquist,1993; Rinehart *et al.*, 2007). Le nom attribué à ces protéines provient de leur découverte chez *D. melanogaster* en réponse à un stress thermique, mais ces protéines sont exprimées en réponse à un large panel de stress chez les insectes. Les différentes Hsp sont définies selon leurs masses moléculaires : Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, etc. (Feder & Hofmann 1999; Gething & Sambrook 1992). Le mode d'action spécifique de chacune de ces Hsp reste à ce jour ambigu au niveau cellulaire. Cependant, il est admis que les Hsp sont sur-exprimées lors de l'exposition à des conditions stressantes et réparent les protéines endommagées ou éliminent celles qui ne peuvent plus être utilisées. Lors de la dessiccation des tissus, les Hsp70 et Hsp90 sont généralement induites et synthétisées en grande quantité par les insectes. Au niveau moléculaire, cette synthèse se traduit par une sur-expression des gènes codant la synthèse des Hsp70 et Hsp90 (Benoit *et al.*, 2010b; Hayward *et al.*, 2004; Rinehart *et al.*, 2006; Siaussat *et al.*, 2013 ; Sinclair *et al.*, 2007). En effet, l'arrêt de l'expression de ces gènes par des méthodes d'ARN interférents ou RNA interference (RNAi) chez *Ae. aegypti* inhibe la dégradation des protéines dénaturées et agrégées, ce qui diminue la longévité des insectes soumis à une dessiccation stressante (Benoit *et al.*, 2010b). D'autres protéines chaperonnes de la famille des Hsp, ne sont pas induite par un état de stress mais sont constitutives. Ces protéines sont nommées *Heat-shock cognate* ou HSC.

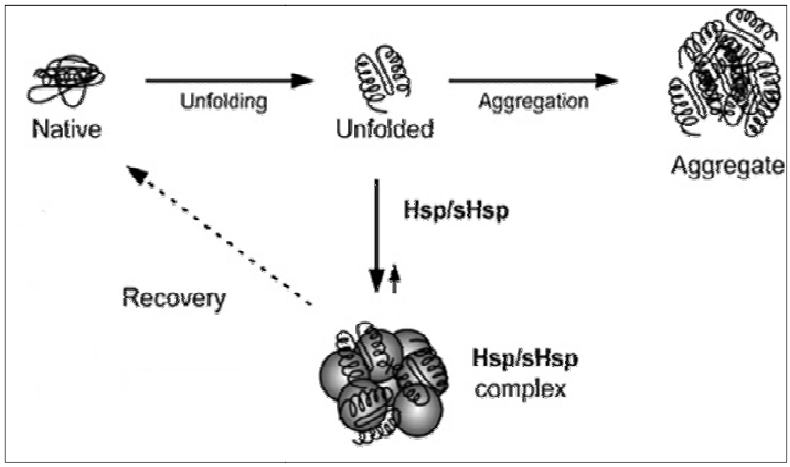


Fig. 9 Représentation schématique du mode d'action des Hsp pour éviter l'agrégation de protéines dénaturées en conditions déshydratantes (Wilhelmus *et al.*, 2007).

Outre les Hsp, d'autres familles de protéines chaperonnes, moins étudiées, existent. Il s'agit des *glucose-regulated proteins* (GRP). Il existe peu de données traitant de l'expression de ces protéines en réponse à la dessiccation des insectes terrestres. Néanmoins, il semblerait que ces protéines soient synthétisées au niveau du réticulum endoplasmique des cellules (Storey & Storey, 2012). Le rôle et le fonctionnement de ces protéines dans la résistance à la dessiccation des insectes restent à être déterminés.

2.4.2. Protéines stabilisatrices des membranes

Outre la dénaturation et l'agrégation des protéines, la dessiccation modifie la structure et la fluidité des membranes plasmiques des cellules. Dans ces conditions, des protéines assurant la stabilité des membranes plasmiques sont synthétisées. Ces protéines sont relativement bien documentées chez les plantes, et incluent des petites protéines de choc thermique telles que les Hsp22 (Bardel *et al.*, 2002) ou encore une protéine nommée *Late Embryonic Abundant protein* (LEA) ou bien LEAm si elle est mitochondriale (Tolleter *et al.*, 2010). L'expression des LEA a été initialement observée au sein des graines déshydratées de coton (Browne *et al.*, 2002). Elle a ensuite été mise en évidence chez le chironome *Polypedilum vanderplanki* (Diptera, Chironomidae) à l'approche de la saison sèche (Kikawada *et al.*, 2008). Les études disponibles chez les plantes montrent que la LEAm change de conformation sous l'effet de la dessiccation, et que ce changement de conformation permet à la protéine d'interagir avec les groupements phosphates des phospholipides des membranes, tels

que la phosphatidylcholine et la cardiolipine. Ces interactions permettent alors une insertion des LEAm au sein même de la membrane afin de protéger les liposomes des effets destructeurs de la dessiccation (**Fig. 10**). A ce jour, des analyses supplémentaires sont requises afin de démontrer le mode d'action des LEA dans la stabilisation des membranes pendant la dessiccation chez les insectes.

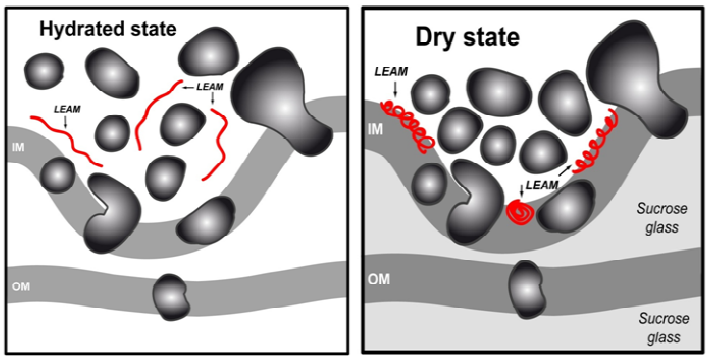


Fig. 10 Représentation schématique du changement de conformation et de l'interaction des LEAm avec les membranes plasmiques en conditions de déshydratation (Tolleter *et al.*, 2010).

2.4.3. Les aquaporines

Les aquaporines constituent une autre famille de protéines étudiées dans le contexte de dessiccation chez les insectes terrestres. Ces protéines, localisées au niveau des membranes plasmiques des cellules, forment des « pores » perméables aux molécules d'eau permettant ainsi leur passage de part et d'autre des membranes (Campbell *et al.*, 2008). Ces protéines sont donc impliquées dans les mécanismes d'ajustements rapides de la balance hydrique et de l'osmolarité entre les espaces inter- et intra-cellulaires. En effet, des études ont montré que les aquaporines jouent un rôle significatif dans la tolérance à la dessiccation chez les insectes (Izumi *et al.*, 2006 ; Philip *et al.*, 2008). Par exemple, chez le chironome *P. vanderplanki*, la dessiccation des tissus corporels stimule l'expression des gènes codant la synthèse des aquaporines (Kikawada *et al.*, 2008). A l'inverse, l'inhibition des gènes codant la synthèse d'aquaporines chez *Eurosta solidaginis* (Diptera, Tephritidae) augmente les dommages cellulaires causés par la dessiccation (Philip *et al.*, 2008).

2.4.4. Les antioxydants

La liste des protéines impliquées dans les réponses à la dessiccation n'est pas exhaustive et d'autres protéines telles que les antioxydants (ex. catalase, superoxyde dismutase, glutathione peroxidase, etc.) sont également impliquées dans la réparation

des effets délétères induits par la dessiccation chez les insectes (Benoit, 2010 ; Teets & Denlinger, 2013). La sur-expression de peptides antioxydants contribue à lutter contre la synthèse de dérivés réactifs de l'oxygène ou *Reactive Oxygen Species* (ROS ; ex. ozone, peroxyde d'hydrogène, etc.) induite par le métabolisme lors de la dessiccation (Storey & Storey, 2007). L'accumulation de ces peptides est bien documentée chez les insectes terrestres soumis à la cryodessiccation (Storey & Storey, 2012). En revanche, l'analyse de ces peptides antioxydants pendant la dessiccation des insectes induite par une hausse des températures reste plus pauvre et est principalement étudiée chez les plantes (Hoekstra *et al.*, 2001 ; White & Torres, 2010).

2.4.5. L'AMP-activated protein kinase

L'*AMP-activated protein kinase* (AMPK) est un candidat prometteur pour l'analyse des réponses des insectes terrestres à la dessiccation (Storey & Storey, 2012). Cette enzyme est en effet un régulateur des processus du catabolisme et de l'anabolisme cellulaire chez les insectes. Par conséquent, l'AMPK pilote la synthèse de protéines et/ou de métabolites impliqués dans la réponse au stress. En condition de stress, la régulation des dépenses énergétiques est importante car cette énergie est primordiale pour résister au stress et doit donc être réallouée en partie aux mécanismes de défenses (Parsons, 1991, 2005). Ainsi, en condition de stress l'expression de cette enzyme inhibe le catabolisme des lipides et des carbohydrates et les dépenses en substrat énergétique tel que l'ATP (Hardie, 2008 ; McBride & Hardie, 2009). Cette inhibition se fait entre autre par des mécanismes de phosphorylation et d'inactivation de l'acetyl-CoA carboxylase.

Une fois encore, l'accumulation de ces peptides en condition de dessiccation a été bien documentée chez les plantes et les mammifères (Cushman & Bohnert, 2000; Horman *et al.*, 2005), mais leur rôle chez les insectes reste toujours à être établi.

2.5. Ajustements de l'osmolalité des fluides corporels

2.5.1. Composés inorganiques

L'osmolalité de l'hémolymphe des insectes est généralement comprise entre 400 et 500 mOsm/kg, mais peut varier de 100 à 1400 mOsm/kg selon les espèces considérées

(Hadley, 1994), et selon leur composition en composés inorganiques (Na^+, K^+, Cl^-, etc.) et organiques (acides gras, acides aminées, polyols, etc.) (Gilles, 2006 ; Sutcliffe, 1962). Chez les invertébrés terrestres, les composés inorganiques Na^+ et Cl^- sont les plus abondants dans le milieu extra-cellulaire et ne pénètrent qu'en petite quantité dans le cytosol des cellules. A l'inverse, les ions K^+ sont fortement représentés dans le milieu intra-cellulaire (Gilles, 2006). En raison de leurs concentrations élevées, les ions Na^+, K^+ et Cl^- interviennent pour 80 à 90% de l'osmolalité des liquides extra-cellulaires, et pour 40 à 75% de l'osmolalité du liquide intra-cellulaire chez la plupart des espèces terrestres d'insectes (Gilles, 2006). Le maintien de l'homéostasie de la balance ionique dans les différents compartiments liquidiens par des mécanismes de diffusion passif et/ou actif (ex. pompes Na^+-K^+-ATPase, Cl^- transporting ATPase, etc.) régule donc le transport des molécules d'eau entre ces compartiments. La diffusion des composés inorganiques entre les compartiments liquidiens est donc impliquée dans le maintien de l'homéostasie de la balance hydrique. En effet, la balance hydrique d'un compartiment est déterminée par les différences d'osmolalité entre deux compartiments contingents (Hadley, 1994), c'est-à-dire par les différences de concentrations des molécules osmotiquement actives exerçant un pouvoir attractif sur les molécules d'eau dans une solution. Ainsi, une augmentation de l'osmolalité d'un compartiment réduit la probabilité de diminution de l'eau au sein de ce compartiment (Chown & Nicholson, 2004). La littérature disponible à ce sujet fait état d'espèces d'insectes capables de séquestrer les sels et autres composés insolubles dans l'hémolymphe au cours d'épisodes de dessiccation (Bayley & Holmstrup, 1999 ; Chown & Nicholson, 2004 ; Holmstrup *et al.*, 2001). Par exemple, chez *Belgica antartica* (Diptera, Chironomidae), l'augmentation de l'osmolalité de l'hémolymphe permet de conserver, au sein de l'hémolymphe, une quantité d'eau suffisante pour le fonctionnement de l'organisme (Levis *et al.*, 2012). De même, chez *Pringleophaga marioni* (Lepidoptera, Tineidae), une augmentation significative de l'osmolalité de l'hémolymphe est observée chez les individus exposés à des conditions déshydratantes (Sinclair & Chown, 2003 ; **Fig. 11**).

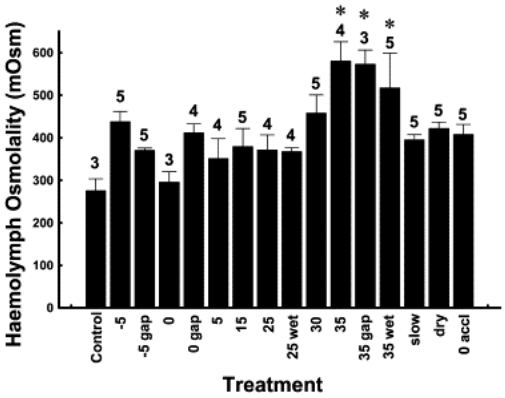


Fig. 11 Osmolalité moyenne (± erreur standard) de l'hémolymphe des chenilles *Pringleophaga marioni* après différents traitements à -5, 0, 5, 15, 25, 30 et 35 °C et des conditions d'humidité variables. Les astérisques désignent une osmolalité significativement différentes des conditions contrôles (10°C) avec P<0.05. D'après Sinclair & Chown (2003).

2.5.2. Composés organiques : les apports de la métabolomique

L'homéostasie de la balance hydrique et osmotique au sein des organismes n'est pas uniquement régulée par les composés inorganiques (Na^+, K^+, Cl^-, etc.), mais également par les composés organiques tels que les polyols, les acides aminés ou encore les acides gras. Parmi ces composés, certains possèdent en plus des qualités d'osmorégulation, des qualités de protection contrebalançant les effets négatifs de la dessiccation au niveau cellulaire (Benoit 2010 ; Goyal *et al.*, 2005 ; Teets & Denlinger, 2013 ; Yancey, 2005). Ces composés, nommés solutés compatibles, n'interférent pas avec le métabolisme des organismes et préservent l'intégrité autour des membranes et protéines (Crowe, 1992). Les avancées techniques en métabolique ont permis un meilleur criblage de ces composés et de leurs actions pendant la dessiccation. L'étude de ces composés en conditions de stress a ainsi été décuplée ces dernières années chez les insectes (Article I, III, VI ; Colinet & Renault, 2014 ; Colinet *et al.*, 2012 ; Hayward, 2014 ; Teets *et al.*, 2012 ; etc.). L'analyse de cette littérature rend compte d'une accumulation de solutés compatibles en réponse à la dessiccation chez de nombreuses espèces d'insectes. Parmi ces composés, les sucres de faible poids moléculaire, tels que le glycérol, le sorbitol ou encore l'inositol et le *myo*-inositol sont les plus communs. En effet, ces composés permettent de préserver les protéines de la dénaturation et les membranes cellulaires de la déstructuration produite par la dessiccation en maintenant les liaisons hydrogènes (Yancey, 2005).

Le type de solutés compatibles accumulés est « espèce dépendant ». Ainsi, tandis que le *myo*-inositol constitue le principal composé accumulé chez *Drosophila montana* (Diptera, Drosophilidae) en conditions de dessiccation (Vesala *et al.*, 2012),

le glycérol et le sorbitol sont accumulés chez *E. solidaginis* (Storey *et al.*, 1981). En plus des sucres à faibles poids moléculaire, d'autres molécules peuvent aussi agir comme solutés compatibles. Par exemple, les larves du moustique *Culex tarsalis* (Diptera, Culicidae) contrebalancent l'effet de la dessiccation par l'accumulation du tréhalose et de la proline dans les compartiments inter- et intra-cellulaires (Patrick & Bradley, 2000). Le tréhalose est également utilisé par les larves de *D. melanogaster* en conditions de dessiccation (Thorat *et al.*, 2012 ; **Fig. 12**). En effet, ce disaccharide non-réducteur contribue à la stabilisation des lipides des membranes plasmiques pendant la dessiccation.

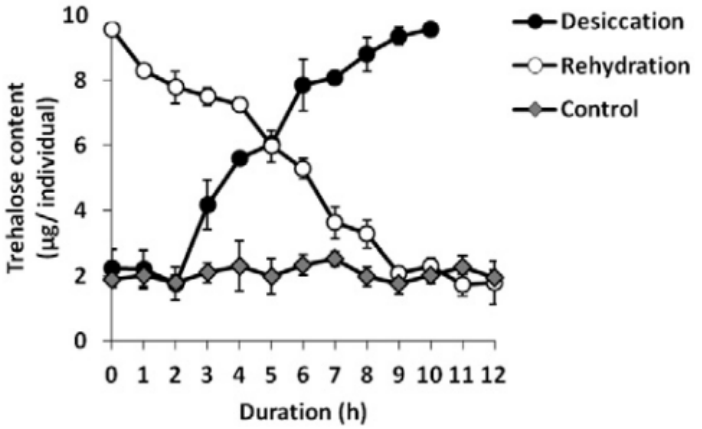


Fig. 12 Concentration moyenne (± erreur standard) du tréhalose pendant la dessiccation et la phase de réhydratation des larves de *D. melanogaster* (Thorat *et al.*, 2012).

Outre les solutés compatibles, la mobilisation et le catabolisme des carbohydrates de réserve stockés dans le corps gras, tel que le glycogène, sont utilisés par les insectes terrestres pendant la dessiccation. En effet, le catabolisme de cette macromolécule est à l'origine d'une synthèse jusqu'à cinq fois sa propre masse en molécules d'eau (Gibbs, 2002; Gibbs *et al.*, 1997; Schmidt-Nielsen, 1990). Bien que cette contribution puisse apparaitre moindre pour la plupart des espèces, elle constitue une stratégie non négligeable pour les organismes exposés à la dessiccation, comme c'est le cas chez les ténébrionidés *Eleodes armata* et *Cryptoglossa verrucosa* (Cooper, 1985). Plus récemment, l'accumulation de cette macromolécule de réserve dans l'hémolymphe des insectes a été observée pendant la dessiccation des larves de *P. vanderplanki* (Mitsumasu *et al.*, 2010), ou encore chez *E. solidaginis* (Williams & Lee, 2008).

Le catabolisme du glycogène est assuré par une protéine phosphorylase (glycogène phosphorylase), dont l'expression est régulée par des hormones adipokinétiques (AKH). Ces hormones ont été largement étudiées pour leur rôle de régulation du métabolisme énergétique dans le contexte du vol chez les insectes (Arrese & Soulages, 2010). Cependant, l'implication des AKH dans un contexte de stress environnemental tel que la dessiccation chez les insectes a été récemment soulevée (Hahn & Denlinger, 2011 ; Isabel *et al.*, 2005). En effet, une relation positive entre la synthèse des AKH et la synthèse du tréhalose dans l'hémolymphe a été suggérée (Isabel *et al.*, 2005). Des études s'avèrent cependant nécessaires afin de valider cette hypothèse (Article II, III).

2.6. Réponses comportementales

Outre les réponses morphologiques, physiologiques et biochimiques, des réponses comportementales permettent également aux espèces d'insectes des milieux arides de limiter la dessiccation de leurs tissus corporels. Une première stratégie consiste à migrer vers un habitat ou micro-habitat temporairement plus favorable pour la biologie de l'organisme (Hadley, 1994). D'après les travaux d'Edney (1977), ce comportement de recherche est régulé par le degré de perturbation de la balance osmotique, et en particulier par l'augmentation de la concentration en ion chlorure (Cl^-) dans l'hémolymphe des organismes. Par exemple, en conditions déshydratantes, la mouches du fruit *D. melanogaster* recherche activement des milieux où l'humidité relative est plus élevée (Prince & Parsons, 1977). Ce comportement de recherche peut s'établir sur de courtes durées. Ainsi, la chenille de l'espèce *Manduca sexta* (Lepidoptera, Sphingidae) s'alimente préférentiellement sur la face inférieure des feuilles de tabac, qui est moins exposée au soleil et où l'humidité est plus importante, pendant les heures les plus chaudes et déshydratantes de la journée (Rowley & Hanson, 2007).

D'autres organismes montrent un comportement d'alimentation excessif afin de combler et/ou d'anticiper les pertes en eau subies suite à une dessiccation. Ce comportement est principalement observé chez les insectes piqueurs-suceurs qui utilisent le nectar, le sang ou d'autres aliments riches en eau pour se réhydrater (Benoit, 2011 ; Benoit *et al.*, 2011).

Enfin, une autre stratégie consiste en l'agrégation d'individus les uns avec les autres dans un milieu confiné pour recréer un micro-habitat avec une humidité relative plus importante (Chown & Nicholson, 2004 ; Yoder *et al.*, 1993). En effet, l'agrégation d'individus augmente l'activité métabolique moyenne au sein du groupe, et augmente ainsi localement l'humidité relative de l'environnement (Yoder *et al.*, 1993). La relation positive entre l'agrégation d'un groupe d'individus et la diminution des pertes en eau corporelle au sein de ce groupe est commune chez les arthropodes (Benoit *et al.*, 2007 ; Doggett *et al.*, 2004 ; Glass *et al.*, 1998 ; Yoder & Smith, 1997). Un des exemples d'agrégation les plus fascinants utilisé pour limiter la dessiccation est celui de l'espèce *Stenotarsus rotundus* (Coleoptera, Endomychidae), où plus de 70 000 individus peuvent se regrouper dans des anfractuosités, à la base de palmiers, lorsque le risque de dessiccation est trop important (Yoder *et al.*, 1993).

2.7. Synthèse schématique des principaux mécanismes de réponses à la dessiccation chez les insectes terrestres

La **Fig. 13** ci-dessous présente un résumé des principaux dommages causés par la dessiccation et des principaux mécanismes de réponse mis en place chez les insectes terrestres. Cette représentation n'est pas exhaustive et fait état des principaux mécanismes de réponse connus et les plus étudiés chez les insectes.

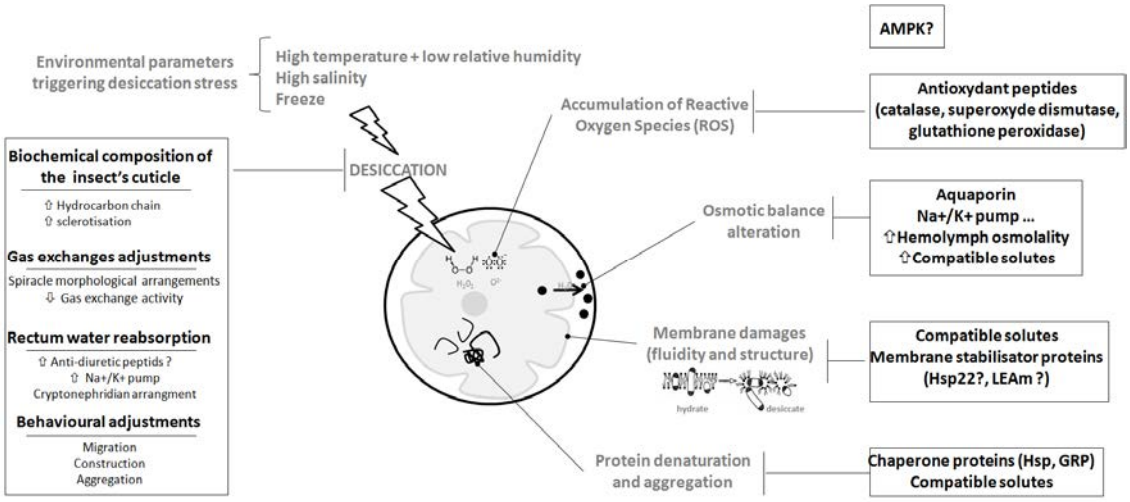


Fig. 13 Construction schématique des principaux dommages causés par la dessiccation (gris) et les principales réponses mis en place par les insectes terrestres (noir).

3. Les moustiques *Anopheles gambiae s.l.* et leurs environnements

3.1. Présentation des moustiques du complexe *Anopheles gambiae s.l.*

3.1.1. Systématique et taxonomie

Les Anophèles sont des moustiques appartenant à l'ordre des Diptères, sous-ordre des Nématocères, famille des Culicidae et sous-famille des Anophelinae. Les Anophèles sont souvent organisés en complexe d'espèces. Par définition, un complexe d'espèces correspond à un ensemble d'organismes semblables sur la base de critères morphologiques, mais différenciés sur la base de critères comportementaux, génétiques et écologiques (Coluzzi *et al.*, 2002). En Afrique sub-saharienne, le complexe d'espèces *Anopheles gambiae sensu lato (s.l.)* représente le groupe taxonomique le plus étudié car il regroupe les plus importants vecteurs de paludisme humain au monde (Fontenille & Simard, 2004). Il compte aujourd'hui huit espèces jumelles qui se distinguent sur la base de critères bio-écologiques, cytologiques, biochimiques et moléculaires (Coetzee *et al.*, 2013 ; Lee *et al.*, 2013 ; **Encadré A**). Les plus importantes, tant par l'étendue de leur aire de distribution sur le continent Africain que par leur importance épidémiologique sont *Anopheles arabiensis, An. coluzzii et An. gambiae sensu stricto (s.s.). Anopheles coluzzii* et *An. gambiae* n'ont que très récemment été différenciées en tant qu'espèces (Coetzee *et al.*, 2013), après avoir longtemps été considérées comme des « formes moléculaires » d'*An. gambiae s.s.*, dénommées S et M, respectivement.

Dans la suite du manuscrit, je n'évoquerai que les espèces *An. coluzzii* et *An. gambiae s.s.* Par ailleurs, cette dernière espèce sera annotée *An. gambiae* pour faciliter la lecture du manuscrit.

ENCADRE A. LE COMPLEXE D'ESPECES *ANOPHELES GAMBIAE S.L.*

Huit espèces jumelles ont été identifiées au sein du complexe d'espèce *Anopheles gambiae s.l.* On distingue alors :

Anopheles amharicus **Hunt, Wilkerson & Coetzee sp.n**. Les moustiques sont principalement zoophiles et présents en Ethiopie (**Fig A4** – rose).

***Anopheles arabiensis* Patton, 1904.** Les larves sont associées aux collections d'eau douce et les moustiques sont à la fois anthropophiles et zoophiles. Ces moustiques sont très impliqués dans la transmission du paludisme en Afrique de l'ouest. Cette espèce présente une très large distribution en Afrique sub-saharienne (**Fig. A1** - rouge).

***Anopheles bwambae* White, 1985.** Les larves sont associées aux collections d'eau salée. Espèce uniquement présente dans les sources chaudes de la forêt de Semliki (parc national à l'est de l'Ouganda ; **Fig. A3** - carré bleu clair).

***Anopheles coluzzii* Coetzee *et al.*, 2013.** Les larves sont associées aux collections d'eau douce et les moustiques sont à la fois anthropophiles et zoophiles. Ces moustiques sont très impliqués dans la transmission du paludisme en Afrique de l'ouest. Cette espèce est présente au niveau de l'Afrique de l'ouest et centrale. L'espèce a été récemment identifiée comme espèce jumelle d'*An. gambiae s.s.,* de ce fait, sa distribution n'a pas encore été modélisée.

***Anopheles gambiae s.s.* Gilles.** Les larves sont associées aux collections d'eau douce et les moustiques sont à la fois anthropophiles et zoophiles. Ces moustiques sont très impliqués dans la transmission du paludisme en Afrique de l'ouest. Cette espèce présente une très large distribution en Afrique sub-saharienne (**Fig. A2** - vert).

***Anopheles quadriannulatus* Theobald, 1911.** Les moustiques sont principalement zoophiles et présents dans le sud-est de l'Afrique (**Fig A4** – jaune).

***Anopheles melas* Theobald, 1903.** Les larves sont associées aux collections d'eau salée. Espèce présente sur les côtes de l'Afrique de l'ouest et centrale (**Fig. A3** - bleu).

***Anopheles merus* Dönitz, 1902.** Les larves sont également associées aux collections d'eau salée. Espèce présente sur les côtes de l'Afrique de l'est et centrale. Présente également à Madagascar (**Fig. A3** - orange).

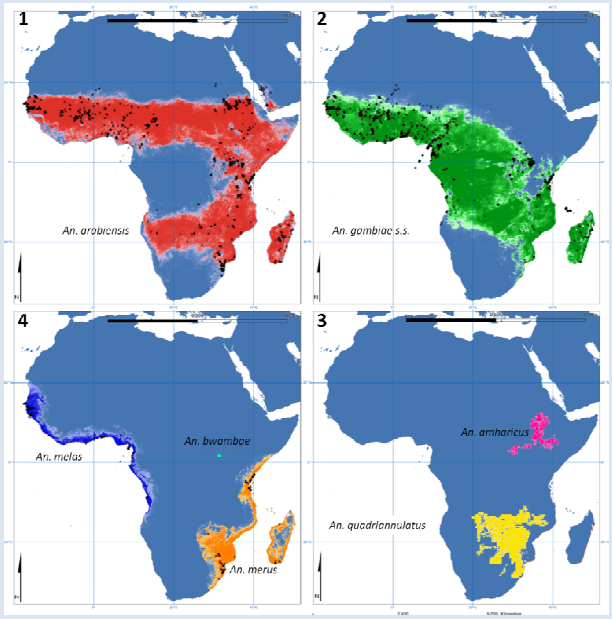


Fig. A Carte de répartition des espèces du complexe *An. gambiae s.l.* sur le continent Africain. Cette figure ne représente pas la distribution d'*An. coluzzii*. D'après Lee *et al.* (2013).

3.1.2. Bio-écologie des Anophèles

Les mâles se nourrissent exclusivement de nectar, jus sucrés et autres exsudats de végétaux. La prise d'aliments sucrés n'est cependant pas limitée aux mâles, puisque les femelles ont également des besoins en ressources énergétiques sucrées, notamment à l'émergence et avant l'accouplement. En effet, les réserves accumulées pendant la vie pré-imaginale étant relativement basses chez l'Anophèle (Fernandes & Briegel, 2005), l'apport en sucre est recherché préférentiellement à l'émergence des imagos (Foster & Takken, 2004). Cet apport est supposé fournir les ressources énergétiques nécessaires aux moustiques pour le développement et la reproduction (Foster, 1995). L'accouplement des moustiques n'a lieu qu'une seule fois au cours de la vie des femelles et se passe dans des essaims au crépuscule ou à l'aube (Charlwood *et al.*, 2002). A l'issue de l'accouplement, la femelle stocke les spermatozoïdes dans une spermathèque, de façon à ce que les œufs puissent être fécondés lors de leur passage dans l'oviducte tout au long de la vie de la femelle. Suite à l'accouplement, les femelles recherchent activement un hôte afin d'y prélever le sang nécessaire au développement des œufs. En effet, les protéines de l'hémoglobine sont une source d'acides aminés essentiels au processus de vitellogenèse et d'oogenèse des femelles. Les processus de digestion du sang et de la maturation ovarienne durent entre 36 et 48 heures (Clements, 2012). A l'issue de cette période, la femelle est dite gravide et prête à pondre.

La séquence repas sanguin, maturation ovarienne et ponte est répétée plusieurs fois au cours de la vie de la femelle. On parle alors de cycle gonotrophique. Par définition, la durée d'un cycle gonotrophique correspond au temps séparant deux pontes successives. Ce cycle est d'environ 48 heures à une température de 25°C chez *An. gambiae*.

Après l'éclosion des œufs, quatre stades larvaires aquatiques séparés par trois mues successives ont lieu. Les larves de moustiques sont considérées comme détritivores bien qu'elles se nourrissent également de micro-organismes vivants (Clements, 2012). A l'issue de ces quatre stades larvaires (8-12 jours selon la température) une pupe est formée. Au cours du stade pupe, de profonds remaniements morphologiques, anatomiques et physiologiques ont lieu afin de permettre à l'insecte

de passer de l'état aquatique à l'état aérien. Les jeunes imagos émergent et restent immobiles au cours des premières heures, afin que le durcissement de la cuticule et le déploiement des ailes s'opèrent. Ils atteignent leur maturité sexuelle 24 à 48 heures après émergence. La longévité des imagos est estimée à environ 20 jours et ne dépasserait pas un mois en conditions naturelles (Gillies & De Meillon, 1968 ; Lehmann & Diabaté, 2008).

Le cycle de vie des moustiques du complexe *An. gambiae s.l.* est représenté par la **Fig. 14** ci-dessous.

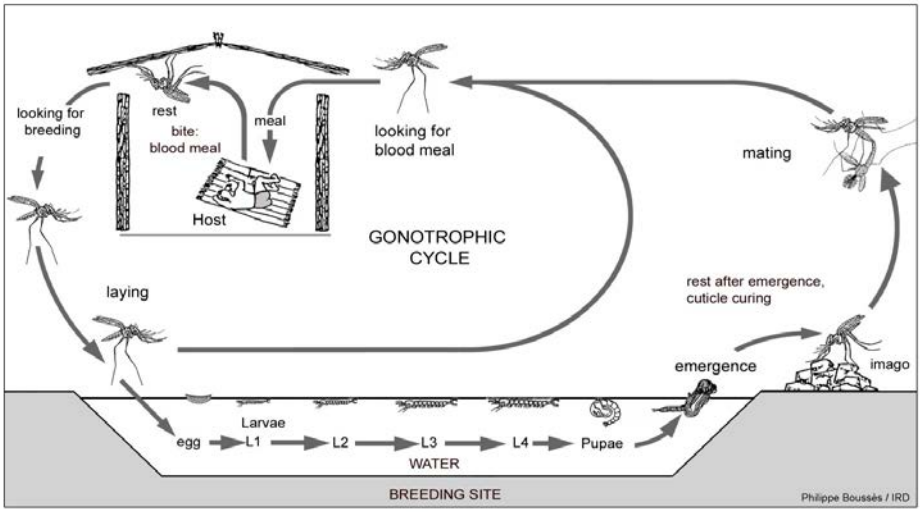


Fig. 14. Cycle de vie des Anophèles.

3.1.3. Importance épidémiologique et méthodes de contrôle

Sur près de 500 espèces d'Anophèles répertoriées dans le monde (Harbach, 2004), environ 70 sont vecteurs du paludisme et 41 sont considérées comme vecteurs à impact majeur sur la santé publique. Le paludisme est une maladie endémique qui sévit dans les régions tropicales et subtropicales de l'Afrique, de l'Amérique du sud et de l'Asie du sud et du sud-est (**Fig. 15**). A l'échelle mondiale, le paludisme constitue une préoccupation majeure de santé publique : 102 pays exposés et >40% de la population mondiale, soit >2 milliards de personnes, vivent dans des régions à risque.

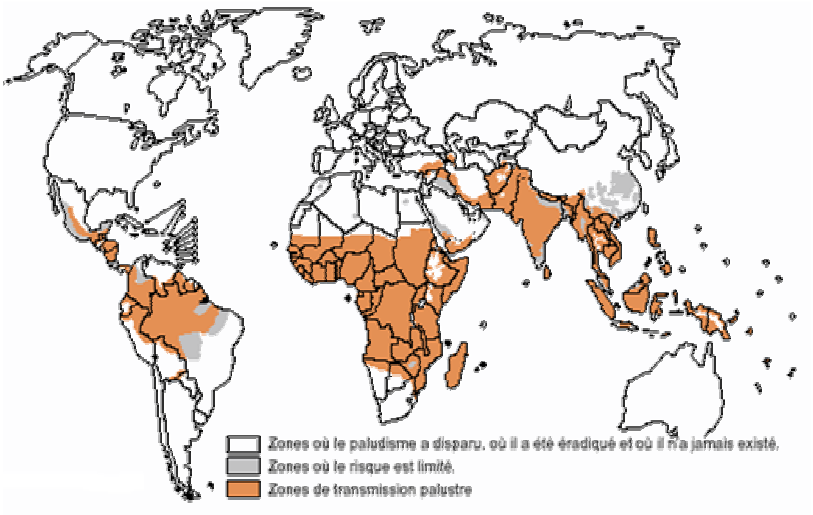


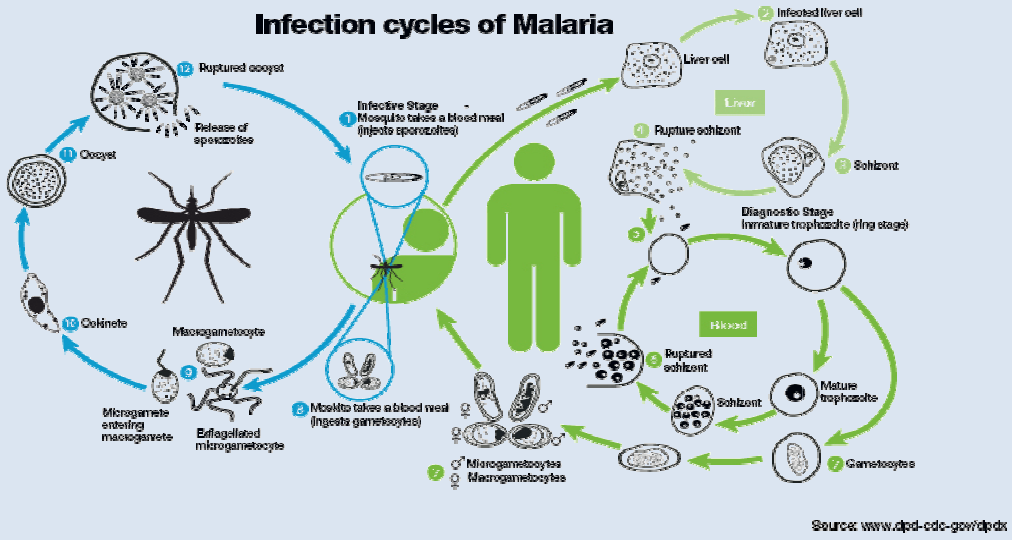
Fig. 15. Cartographie des pays ou territoires où il existe un risque de transmission palustre, d'après l'organisation mondiale de la santé, en 2011. En blanc : zones où le paludisme a disparu ou n'a jamais existé. En gris : zone où le risque est limité. En orange : zones de transmission palustre.

Le continent Africain est particulièrement touché par ce fléau. D'après l'organisation mondiale de la santé, 627 000 décès dus au paludisme ont été recensés sur le continent, en 2012 (WHO, 2013). Bien que les campagnes de lutte montrent des avancées significatives dans le recul de la maladie, celles-ci ne sont pas suffisantes et l'apparition de mécanismes de résistance aux méthodes de lutte actuelles risque d'aggraver le risque sanitaire.

La transmission du paludisme implique trois acteurs : le parasite (*Plasmodium sp.*), le vecteur (Anophèles) et l'Homme (**Encadré B**). Les méthodes de lutte contre le paludisme s'articulent autour de trois volets d'actions : (1) la lutte contre le parasite, (2) la lutte anti-vectorielle et (3) le développement de stratégies vaccinales. La lutte contre le parasite repose essentiellement sur des méthodes de chimiothérapie anti-plasmodique des malades et de chimioprophylaxie des personnes à risques élevées (ex. femmes enceintes, enfants <5 ans et personnes provenant de zones sans présence du paludisme). Cependant, ces méthodes restent souvent coûteuses et inaccessibles aux plus nécessiteux. La lutte anti-vectorielle vise à contrôler les populations de vecteurs pour prévenir l'infection. Ces méthodes reposent actuellement sur l'utilisation d'insecticides imprégnés sur les moustiquaires ou par aspersion intra-domiciliaire, et dans une moindre mesure, la sensibilisation pour la suppression de micro-habitats (collection d'eau) favorables au développement des stades larvaires. Aucune méthode de stratégie vaccinale n'est à ce jour disponible, et l'ensemble de ces méthodes de lutte

est menacé par l'utilisation excessive d'insecticides et le développement de mécanismes de résistance tant au niveau des vecteurs qu'au niveau des parasites (Dabire *et al.*, 2009 ; Diabaté *et al.*, 2004 ; Hargreaves *et al.*, 2000; Namountougou *et al.*, 2012 ; N'Guessan *et al.*, 2007). La mise en place de stratégies de lutte anti-vectorielle efficaces, ciblées et respectant l'environnement est aujourd'hui nécessaire. Toutefois, ces stratégies nécessitent une meilleure caractérisation de l'écologie et de la physiologie des vecteurs du paludisme dans leurs environnements, et en particulier pendant les conditions déshydratantes de la saison sèche, où les vecteurs sont les plus à même d'être contrôlés (Fontenille & Simard, 2004).

ENCADRE B. TRANSMISSION DU PALUDISME



Phase hôte humain : lors de la prise d'un repas de sang, l'Anophèle femelle infectée par l'agent du paludisme, inocule les sporozoïtes dans l'hôte humain **(1)**. Les sporozoïtes infectent alors les cellules hépatiques de l'hôte **(2)** et, à maturité, deviennent des schizontes **(3)**, qui éclatent et libèrent des mérozoïtes **(4)**. Les mérozoïtes infectent les hématies et forment des trophozoïtes **(5)**. Les trophozoïtes de forme annulaire re-deviennent des schizontes, qui éclatent et libèrent de nouveau mérozoïtes qui ré-infectent de nouvelles hématies **(6)**. Certains parasites prennent des formes sexuées différenciées pendant le stade trophozoïte, formant les micro et macrogamétocytes **(7)**.

Phase vecteur : Au cours d'un repas de sang, l'Anophèle femelle ingère les formes sexuées **(8)** du parasite qui se différencient en gamètes mâles et femelles dans l'estomac du vecteur **(9)**. Ces derniers fusionnent, forment un ookinète mobile **(10)** qui traverse la paroi de l'estomac et s'enkyste, formant alors un oocyste **(11)**. L'oocyste mature éclate, libérant des milliers de sporozoïtes qui migrent jusqu'aux glandes salivaires du moustique et seront injectés à un nouvel hôte lors de la prochaine piqûre.

En Afrique sub-saharienne, où plus de 80% des cas surviennent, le paludisme est essentiellement transmis par *An. arabiensis*, *An. coluzzii* et *An. gambiae* (Coetzee et al., 2000 ; Fontenille *et al.*, 2003). Néanmoins, alors qu'*An. arabiensis* présente un comportement d'alimentation anthropophile et zoophile, les femelles *An. coluzzii* et *An. gambiae* sont hautement anthropophiles et s'alimentent donc sur des hôtes humains (Coluzzi et al., 1979). De ce fait, le contrôle des populations d'*An. coluzzii* et *An. gambiae* se révèle d'une grande importance afin de contrôler la transmission du paludisme aux hommes en Afrique sub-saharienne.

3.2. Les femelles *Anopheles coluzzii et An. gambiae* et la saison sèche

3.2.1. Ecologie larvaire et phénologie saisonnière

Les exigences en ensoleillement des stades pré-imaginaux des moustiques *An. coluzzii* et *An. gambiae* ne leur permettent pas de vivre en sous-bois. En revanche, leur présence est abondante au sein et aux alentours des villes et villages et le long des routes et pistes dégagées des régions forestières. Par ailleurs, ces espèces sont présentes depuis le niveau de la mer, mais ne se retrouvent que rarement au-dessus de 1000 mètres d'altitude.

Des différences au niveau de l'écologie larvaire des femelles *An. coluzzii* et *An. gambiae* ont été mises en évidence. En effet, les larves d'*An. gambiae* se développent dans les petites collections d'eau peu profondes, ensoleillées et dépourvues de végétation (fossés, ornières, empreintes, petites mares, flaques, etc.) dont la mise en eau dépend des précipitations. Les larves sont donc présentes en grand nombre pendant la saison des pluies dans les savannes Africaines (Baldet et al., 2003 ; Costantini et al., 2009 ; Diabate *et al.*, 2002, 2004). Les larves d'*An. coluzzii*

exploitent ces mêmes gîtes larvaires et sont souvent trouvées en sympatrie avec les larves d'*An. gambiae*. Mais, elles sont également capables de se développer dans des collections d'eau permanentes, généralement d'origine anthropique (barrages, périmètres irrigués pour l'agriculture,…) qui persistent tout au long de l'année (Baldet et al. 2003 ; Gimonneau et al. 2012a ; Touré *et al.*, 1994, 1998; **Fig. 16 & 17**). Il a été suggéré que la capacité des larves d'*An. coluzzii* à exploiter des collections d'eau permanentes est liée à une meilleure compétitivité et une stratégie anti-prédation plus efficace que les larves d'*An. gambiae* dans ces milieux riches en faune entomophage (Gimonneau *et al.*, 2010, 2012a, 2012b).

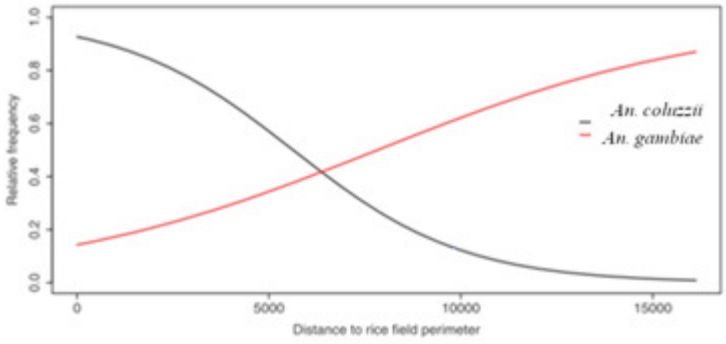


Fig. 16. Fréquence relative des larves d'*An. coluzzii* (noir) et *An. gambiae* (rouge), en fonction de la distance aux rizières (gites larvaires anthropisés) à Bama, Burkina-Faso. D'après Gimonneau *et al.* (2012a).

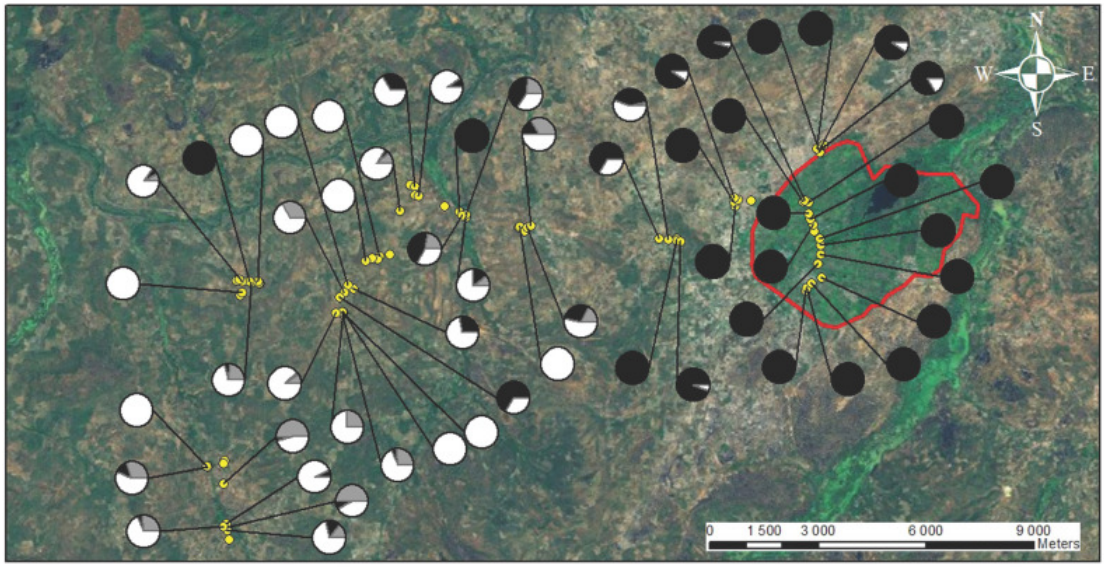


Fig. 17. Cartographie de la région de Bama (sud-ouest du Burkina-Faso, Afrique de l'ouest) illustrant la fréquence relative et la distribution en 2009 des populations d'*An. gambiae* (blanc), *An. coluzzii* (noir) et *An. arabiensis* (gris) au sein de la savane aride (gites temporaires) et de la zone de riziculture irriguée (gîtes larvaires permanents, zone cerclée de rouge). Gimonneau *et al.* (2012a).

La ségrégation des espèces *An. coluzzii* et *An. gambiae* selon les caractéristiques écologiques de leurs gîtes larvaires (collection d'eau) peut induire des différences bio-écologiques entre les deux espèces et/ou populations (Fontenille *et al.*, 1997; Touré *et al.*, 1998), et notamment dans les régions caractérisées par une saison sèche prononcée, comme c'est le cas dans les savanes d'Afrique de l'ouest. La saison sèche se caractérise par une élévation des fluctuations thermiques journalières liées à des températures plus chaudes en journée et plus fraiches pendant la nuit, un vent sec et chaud, de rares précipitations et une importante diminution de l'humidité relative. La **Fig. 18** et le **Tableau 2** résument l'ensemble des variations climatiques et écologiques induites par la saison sèche au Burkina-Faso (Afrique de l'ouest). Dans un tel contexte climatique, les collections d'eau naturelles s'assèchent rapidement dès le début de la saison sèche et le risque de dessiccation des insectes est important. Ces points d'eau se rempliront à nouveau au début de la prochaine saison des pluies.

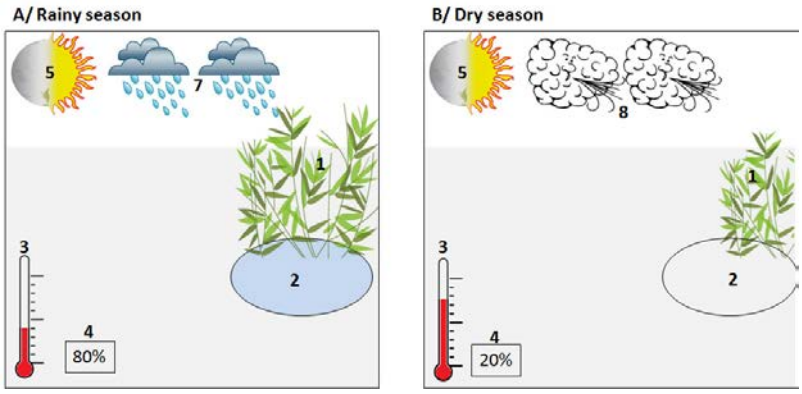


Fig. 18 Construction schématique des principales différences climatiques et écologiques entre la saison des pluies (A) et la saison sèche (B) au Burkina-Faso (Afrique de l'ouest). D'après Huestis & Lehmann (2014). Voir le **Tableau 2**.

Tableau 2 Tableau résumant les principales différences climatiques et écologiques entre la saison des pluies (A) et la saison sèche (B) au Burkina-Faso (Afrique de l'ouest) présentées dans la **Fig. 18**.

	Rainy season (A)	**Dry season (B)**
1/ Vegetation	Available	Moderately available
2/ Water pond	Available	Rare
3/ Temperature	Stable et moderated (25-35 °C)	Instable et hot (20-40 °C)
4/ Relative humidity	Hight (70-95%)	Low (15-25%)
5/ Photoperiod	12:12 (L:D)	13,25:11,75 (L:D)
6/ Wind	Soft and rare	Frequent, hot and dry
7/ Rainfall	Frequent	Rare or absent

Il a été montré que les stades pré-imaginaux (œufs, larves et pupes) ne sont pas capables de survivre sans présence d'eau plus de quelques jours, et que leur degré de résistance à la dessiccation est extrêmement faible (Beier *et al.*, 1990; Koenraadt & Takken, 2003; Minakawa *et al.*, 2001). Par conséquent, les populations de moustiques *An. coluzzii* et *An. gambiae* suivent la dynamique de présence des collections d'eau dans l'environnement (Craig *et al.*, 1999; Patz *et al.*, 1998). Les populations de moustiques *An. coluzzii* exploitant des gîtes larvaires permanents montrent donc des densités peu fluctuantes d'une saison à l'autre, alors que les populations de moustiques *An. coluzzii* et *An. gambiae* exploitant des gites larvaires temporaires (dont la présence dépend des précipitations) montrent des densités fluctuantes. En effet, ces moustiques « disparaissent » au début de la saison sèche (refs. **Fig. 19**).

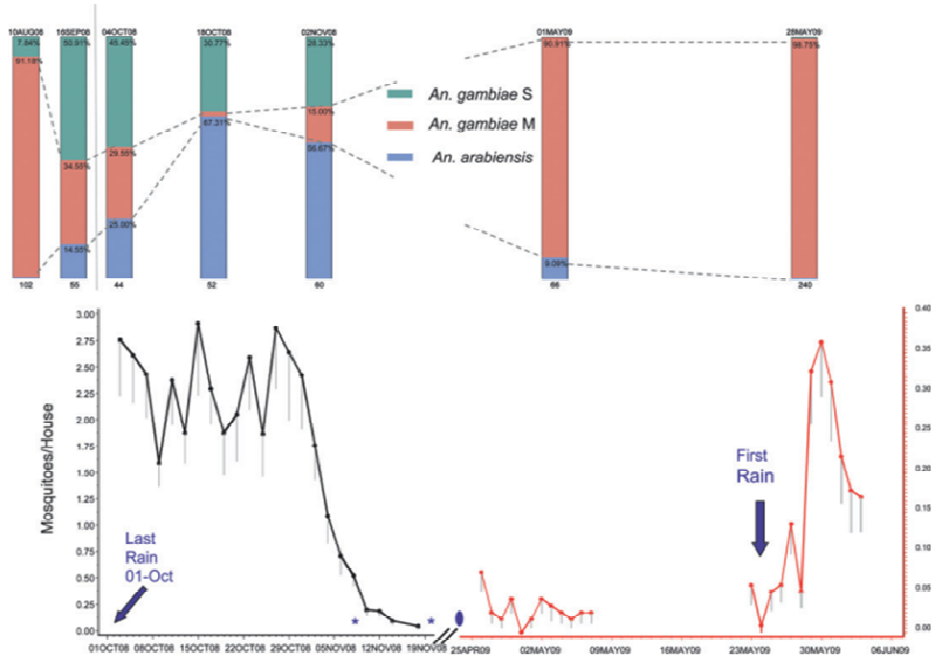


Fig. 19 Abondance (bas ; en nombre moyen de moustiques par maison) et fréquences relatives (haut ; en %) des moustiques *An. coluzzii* (*An. gambiae* M – rouge), *An. gambiae* (*An. gambiae* S – vert) et *An. arabiensis* (bleu) du début de la saison des pluies de 2008 au début de la saison des pluies de 2009, à Thierola, Mali (Afrique de l'ouest). En noir : abondance des moustiques échantillonnés dans les habitations pendant la saison des pluies. En rouge : abondance des moustiques échantillonnés dans les habitations et/ou à proximité pendant la saison sèche (Lehmann *et al.*, 2010).

3.2.2. Mécanismes de réponse des moustiques aux conditions de la saison sèche

Malgré les importantes incidences épidémiologiques des moustiques *An. coluzzii* et *An. gambiae* dans les régions où le paludisme sévit, comment ces vecteurs résistent et survivent aux conditions déshydratantes de la saison sèche reste encore méconnu (Huestis & Lehmann, 2014). Résoudre cette problématique constitue alors un réel défi afin d'établir des méthodes de contrôle innovant de ces vecteurs lors de la saison sèche, pendant laquelle les moustiques semblent les plus fragiles. Les études de génétique et de dynamique des populations menées à ce sujet au cours de la dernière décennie ont tout de même permis de proposer l'existence de deux stratégies de réponses potentielles chez ces insectes : une stratégie de migration (Adamou *et al.* 2011; Huestis & Lehmann, 2014; Lehmann *et al.*, 2010) et une stratégie de diapause estivale nommée estivation[1] (Adamou *et al.*, 2011; Lehmann *et al.*, 2010, 2014). D'autre part, suite aux différences observées au niveau de la bio-écologie et de la phénologie saisonnière d'*An. coluzzii* et *An. gambiae*, ces études suggèrent des stratégies de réponse différentes entre les deux espèces.

Les récents travaux menés par Adamou *et al.* (2011) montrent qu'après un traitement par aspersion de pyréthrinoïdes (insecticides) tout au long de la saison sèche au sein de plusieurs villages de la région du Sahel, la densité des populations des moustiques *An. gambiae* au début de la saison des pluies n'est pas différente de celle de l'année passée où le traitement a été opéré (**Fig. 20**). Bien que ces résultats puissent être la conséquence de mécanismes de résistance aux pyréthrinoïdes chez *An. gambiae* (Chouaïbou *et al.*, 2008 ; Dabiré *et al.*, 2009), les auteurs suggèrent une extinction locale des populations d'*An. gambiae* dans ces zones pendant la saison sèche, puis une recolonisation au début de la saison des pluies. L'hypothèse avancée est donc que les populations d'*An. gambiae* s'éteignent localement au début de la saison sèche puis recolonisent les milieux au début de la saison des pluies par des phénomènes de migration depuis des zones de refuge aux conditions environnementales plus clémentes. Cependant, pourquoi ces moustiques disparaissent au début de la saison sèche restent inexpliqué.

[1]L'estivation est la forme de diapause associée aux hautes températures et basses conditions d'humidité relatives dans les régions tropicales et subtropicales. Elle induit une amélioration des mécanismes de résistance et de tolérance aux hautes températures et à la dessiccation (Navas & Carvalho, 2009).

A l'inverse, le traitement aux pyréthrinoïdes, mis en place par Adamou *et al.* (2011), réduit de 30% la densité de populations d'*An. coluzzii* au début de la saison des pluies comparé à des villages témoins sans traitement (**Fig. 20**). Ce dernier résultat suggère alors que les populations d'*An. coluzzii* restent dans ou à proximité des villages pendant la saison sèche. En accord avec ces résultats, les travaux menés par Lehmann *et al.* (2010, 2014), au Mali, montrent la présence de populations locales permanentes et génétiquement stables de moustiques *An. coluzzii* tout au long de l'année. De plus, des études de « marquage-lâcher-recapture » ont montré qu'une femelle *An. coluzzii*, marquée en fin de saison des pluies, pouvait survivre tout au long de la saison sèche et être capturée de nouveau au début de la saison des pluies suivante (Lehmann *et al.*, 2010), soit 7 mois après. Il a alors été proposé que de telles femelles seraient capables de développer une stratégie d'estivation pendant la saison sèche (Denlinger & Armbruster, 2014 ; Huestis & Lehmann, 2014 ; Mamai *et al.*, 2014). Des données plus anciennes et controversées suggéraient déjà la mise en place d'une telle stratégie de diapause estivale chez des femelles du complexe (Holstein, 1954 ; Omer & Cloudsley-Thompson, 1970). Ces études ont par ailleurs montré que seules les femelles étaient échantillonnées au cours de la saison sèche, aucun mâle n'ayant été observé. Ainsi, la stratégie d'estivation ne concernerait que les femelles et consisterait en une réduction de l'activité métabolique et du développement ovarien (Denlinger & Armbruster, 2014).

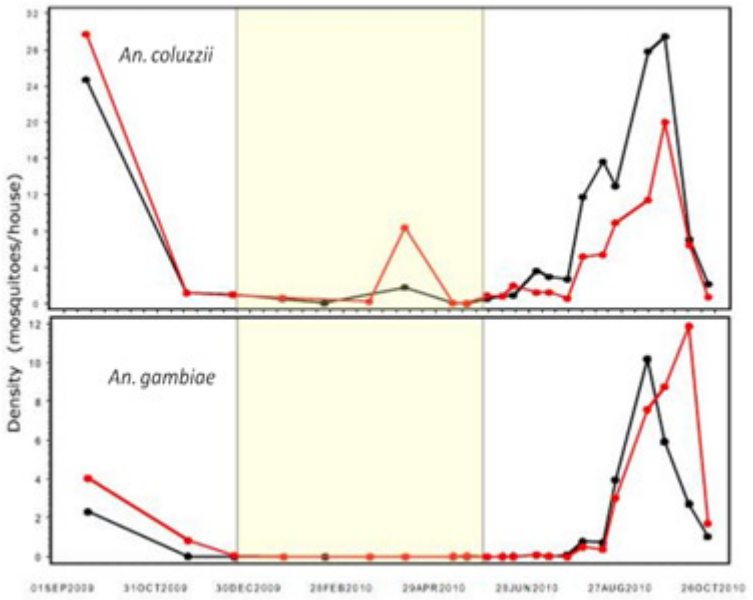


Fig. 20 Abondance moyenne des moustiques *An. coluzzii* (haut) et *An. gambiae* (bas) de septembre 2009 à octobre 2010 dans un village malien. En noir : abondance des moustiques dans les villages « contrôles » non traités. En rouge : abondance des moustiques dans les villages traités aux pyréthrinoïdes. La partie jaune représente l'étendue de la saison sèche (décembre à mai). D'après Adamou *et al.* (2011).

4. Problématique et démarches expérimentales du travail de thèse

Les conditions drastiques de la saison sèche exposent les moustiques Anophèles des régions sub-sahariennes à des conditions environnementales déshydratantes. Des ajustements, à différents niveaux d'intégration, de la biologie de ces espèces sont nécessaires pour y faire face (chapitre I- Section 2). Différentes stratégies semblent avoir émergées chez les populations d'*An. coluzzii* et *An. gambiae*, présentes au Burkina-Faso, de manière à faire face aux contraintes environnementales de la saison sèche. Ainsi, alors que les données issues de la littérature suggèrent que les spécimens d'*An coluzzii* élaborent une stratégie de dormance estivale (estivation), les adultes d'*An gambiae* disparaissent des abords des collections d'eau au début de la saison sèche et recolonisent ces habitats au début de la saison des pluies. Cette phénologie saisonnière suggère une stratégie de dispersion/migration des femelles au début de la saison sèche et au début de la saison des pluies (Adamou *et al.*, 2011 ; Huestis & Lehmann, 2014 ; Lehmann *et al.*, 2010, 2014). Néanmoins, ces hypothèses apparaissent encore fragiles à l'heure actuelle, en raison de l'impossibilité de trouver les microhabitats dans lesquels se situeraient les formes estivantes d'*An. coluzzii* ou encore les zones de refuges d'*An. gambiae* pendant la saison sèche. De plus, ces hypothèses sont construites sur la base d'études indirectes des stratégies de survie des femelles Anophèles, comme l'analyse de la génétique et de la dynamique des populations de ces espèces dans leurs milieux naturels (Adamou *et al.*, 2011 ; Lehmann *et al.*, 2010, 2014). Pourtant, l'établissement d'une stratégie d'estivation et/ou de dispersion/migration des femelles Anophèles en réponse aux conditions de la saison sèche doit être caractérisé par des ajustements au niveau physiologique et morphologique, dont les bases sont bien documentées chez les insectes (Danks, 2000; Denlinger & Armbruster, 2014 ; Drake, 1995 ; Zera *et al.*, 1997). Néanmoins, ces analyses restent rares chez les femelles Anophèles, et les quelques études menées jusqu'à présent ont porté sur des femelles prélevées directement en milieux naturels (Huestis *et al.*, 2011, 2012). Ces études, bien qu'utiles, introduisent néanmoins une forte variabilité expérimentale, en raison des différents statuts physiologiques des femelles (âge, sexe, statut trophique et reproducteur, etc.) qui multiplie fortement le

nombre de phénotypes observé. En raison de la difficulté de caractérisation des stratégies exactes mises en place par les femelles *An. coluzzii* et *An. gambiae* en conditions naturelles lors de l'entrée en saison sèche, de nouvelles expériences en conditions contrôlées représentent une alternative judicieuse, et complémentaire des approches menées en conditions naturelles. L'arsenal d'approches expérimentales permettant de mesurer les ajustements écophysiologiques et morphologiques sur ces espèces, dont l'élevage est parfaitement maitrisé, devrait permettre de faire progresser nos connaissances sur ce modèle biologique. **Ce travail de thèse a pour but d'évaluer et de comparer l'ajustement des caractères écophysiologiques et morphologiques des femelles *An. coluzzii* et *An. gambiae* exposées expérimentalement aux conditions drastiques de la saison sèche.**

4.1. Ecophysiologie comparative des réponses écophysiologiques à l'arrivée de la saison sèche chez les Anophèles (Chapitre II)

4.1.1. Résistance à la dessiccation et profilage métabolique et protéomique des femelles Anophèles (Article I)

Des approches expérimentales on tout d'abord été menées afin d'évaluer si l'exposition des femelles *An. coluzzii* et *An. gambiae* aux conditions climatiques du début de la saison sèche influencent leurs capacités de résistance à la dessiccation. Des analyses comparatives de la survie et des pertes en eau corporelles ont été réalisées. Ces analyses ont été effectuées sur des femelles expérimentalement exposées à des conditions simulant la saison des pluies et le début de la saison sèche, puis exposées à des conditions très arides dès l'émergence des adultes. De récents travaux suggèrent une plasticité physiologique chez les femelles Anophèles selon les conditions d'élevage perçues pendant leur développement (Mamai *et al.*, 2014). Nous supposons que cette plasticité confère une meilleure valeur adaptative aux femelles, quelque soit les conditions environnementales perçues. Ainsi, nous posons l'hypothèse que les femelles élevées en condition du début de la saison sèche expriment des capacités de résistance à la dessiccation plus grandes que celles élevées en condition de saison des pluies. Nous supposons également que cette meilleure capacité de résistance est induite par des ajustements rapides, à l'émergence des femelles, de leurs caractères

physiologiques en réponse aux conditions drastiques de la saison sèche. En effet, les premières heures après l'émergence des imagos, les organismes passent d'un stade larvaire aquatique à un stade imaginal aérien où ils sont soumis à un grand risque de dessiccation. **Nous avons exploré les ajustements des caractères écophysiologiques (métabolomiques et protéomiques) suivant l'émergence des femelles *An. coluzzii* et *An. gambiae* en réponse aux conditions contraignantes de la saison sèche**. Cette première analyse exploratoire a été menée à l'aide de profilages métaboliques (GC-FID, UPLC) et protéiques (2D-DIGE) à grande échelle, chez des femelles Anophèles exposées aux conditions de la saison des pluies et du début de la saison sèche, et a permis d'identifier plusieurs marqueurs physiologiques spécifiques de la réponse des organismes exposés aux conditions de la saison sèche qui ont ensuite servies de base pour la mise en place d'analyses plus poussées dans la poursuite de ce travail.

4.1.2. Ajustements du métabolisme énergétique chez les femelles *An. coluzzii* et *An. gambiae* (Article II)

La régulation hormonale du métabolisme énergétique des insectes est assurée par les hormones adipokinétiques (AKH). La sécrétion d'AKH est ainsi connue pour faciliter la mobilisation et le transport des réserves énergétiques dans un contexte de maintien du vol chez les insectes (Aresse & Soulages, 2010). Une surexpression de ces hormones devrait donc être associée à une forte activité du métabolisme énergétique et de meilleures capacités à disperser chez les femelles Anophèles – notamment *An. gambiae* – au début de la saison sèche. Par ailleurs, des variations de l'activité métabolique sont attendues en relation avec les variations de l'expression des AKH et les mécanismes de survie des femelles. Par exemple, les stratégies de dormance sont connues pour être caractérisées par une dépression de l'activité métabolique des insectes (Hahn & Denlinger, 2007, 2011). Les femelles *An. coluzzii* devraient donc montrer une réduction de l'activité métabolique au début de la saison sèche. **Ce deuxième travail porte sur l'ajustement de l'activité métabolique et la régulation hormonale du métabolisme énergétique, dont les variations sont des marqueurs potentiels des mécanismes de survie des femelles *An. coluzzii* et *An. gambiae*, en réponse aux conditions drastiques de la saison sèche**. La régulation hormonale du

métabolisme énergétique a été analysée à travers l'expression des gènes codant la synthèse des hormones adipokinétiques, et l'activité métabolique via la mesure de la quantité de dioxyde de carbone émise par les individus (Lighton & Fielden, 1995; Rourke, 2000; Terblanche *et al.*, 2005). Par ailleurs, pour la première fois, les réponses des femelles *An. coluzzii* ont été analysées et comparées en fonction de leurs microhabitats d'origine, selon qu'elles exploitent des gîtes larvaires permanents ou temporaires.

4.2. Variabilité de la plasticité phénotypique chez *An. coluzzii* (Chapitre III)

4.2.1. Variabilité de la plasticité physiologique des femelles *An. coluzzii* (Article III)

Les résultats obtenus montrent des ajustements du métabolisme des femelles *An. coluzzii* différents selon que les femelles exploitent des collections d'eau permanentes ou seulement présentes pendant la saison des pluies (Article II). Ces résultats suggèrent l'existence de mécanismes d'adaptation/acclimatation des femelles *An. coluzzii* aux conditions locales de leur environnement. Nous supposons donc que des différences au niveau des caractères écophysiologiques et morphologiques des insectes seront observables entre les différentes populations d'*An. coluzzii*, notamment au début de la saison sèche.

D'autre part, les femelles d'*An. coluzzii* expriment différentes capacités d'estivation, au début de la saison sèche, selon qu'elles exploitent des habitats où les collections d'eau sont maintenues ou non pendant la saison sèche (Yaro *et al.*, 2012). Des signatures physiologiques différentes au niveau du métabolisme énergétique, devraient donc être observées entre les populations d'*An. coluzzii* à l'approche de la saison sèche. **Nous avons analysé la variabilité des réponses écophysiologiques de différentes populations d'*An. coluzzii* à l'arrivée de la saison sèche**. Nous avons exploré les variations des teneurs en réserves corporelles (protéines, lipides et glycogène), des profils métaboliques (acides aminés, intermédiaires du cycle de Krebs, polyols, carbohydrates, etc.) et de la régulation hormonale du métabolisme énergétique (AKH) des femelles exposées aux conditions de la saison des pluies et du début de la saison sèche. Nous supposons que cette exploration permettra d'identifier des

marqueurs physiologiques de l'adaptation/acclimatation des femelles *An. coluzzii* aux conditions locales de leur environnement pendant la saison sèche. Quatre populations prélevées dans des habitats où les collections d'eau sont permanentes ou temporaires, au nord et au sud du Burkina-Faso, ont été retenues pour tester cette hypothèse. Le nord et le sud du Burkina-Faso sont caractérisés par des différences de sévérité de la saison sèche. Ainsi la disparition des collections d'eau, au début de la saison sèche, au nord du Burkina-Faso, où les conditions de saison sèche sont plus drastiques, doit induire des réarrangements physiologiques plus marqués que chez les populations du sud du Burkina-Faso.

4.2.2. Temps de développement pré-imaginal et plasticité morphologique des populations d'*An. coluzzii* (Article IV)

Si les différences observées au niveau physiologique entre les populations d'*An. coluzzii* résultent d'une adaptation/acclimatation aux conditions locales de leur environnement, des changements phénotypiques seront mesurables à d'autres échelles d'analyse. Des changements du temps de développement et de la morphologie de ces moustiques, qui sont deux indicateurs connus du niveau d'adaptation locale des populations d'insectes (Couret *et al.*, 2014; Czarnoleski *et al.*, 2013; Damos & Savopoulou-Soultani, 2012; Dujardin, 2008, 2011), sont alors attendus entre les populations d'*An. coluzzii*. Parmi ces changements morphométriques, les variations de la forme et de la taille des ailes des moustiques sont influencées par les conditions environnementales perçues par les organismes pendant le développement (Andersen *et al.*, 2005; Ayala *et al.*, 2012 ; Morales Vargas *et al.*, 2013; Soto *et al.*, 2006). **C'est pourquoi, nous avons testé la variabilité des réponses développementales et morphologiques des populations d'*An. coluzzii* associées (1) aux conditions environnementales de la saison sèche et (2) aux caractéristiques locales des habitats des femelles**. Cette étude a été menée sur les quatre populations d'Anophèles utilisées dans l'étude précédente, et une colonie de laboratoire considérée comme un groupe externe de comparaison. Les femelles de chaque population ont été élevées dans des conditions reproduisant la saison des pluies et le début de la saison sèche. Le temps de développement larvaire (de l'éclosion à l'émergence), la taille, la surface et

la forme de l'aile droite des femelles ont ensuite été analysés. Nous supposons que des modifications du temps de développement, conduisant à des variations géo-morphométriques des ailes des femelles Anophèles, seront observées selon les conditions d'élevage des femelles (Ayala *et al.*, 2012 ; Czarnoleski *et al.*, 2013; Damos & Savopoulou-Soultani, 2012 ; Morales Vargas *et al.*, 2013). La direction de ces changements doit également varier selon l'origine géographique des femelles, notamment au début de la saison sèche. Ces résultats suggéreraient une plasticité phénotypique des femelles en réponse aux conditions locales de leur environnements (Dujardin *et al.*, 2008, 2011).

4.3. Synthèse schématique de l'intégration des différentes études menées

La **Fig. 21** ci-dessous schématise la démarche expérimentale utilisée dans le cadre de ce travail de thèse.

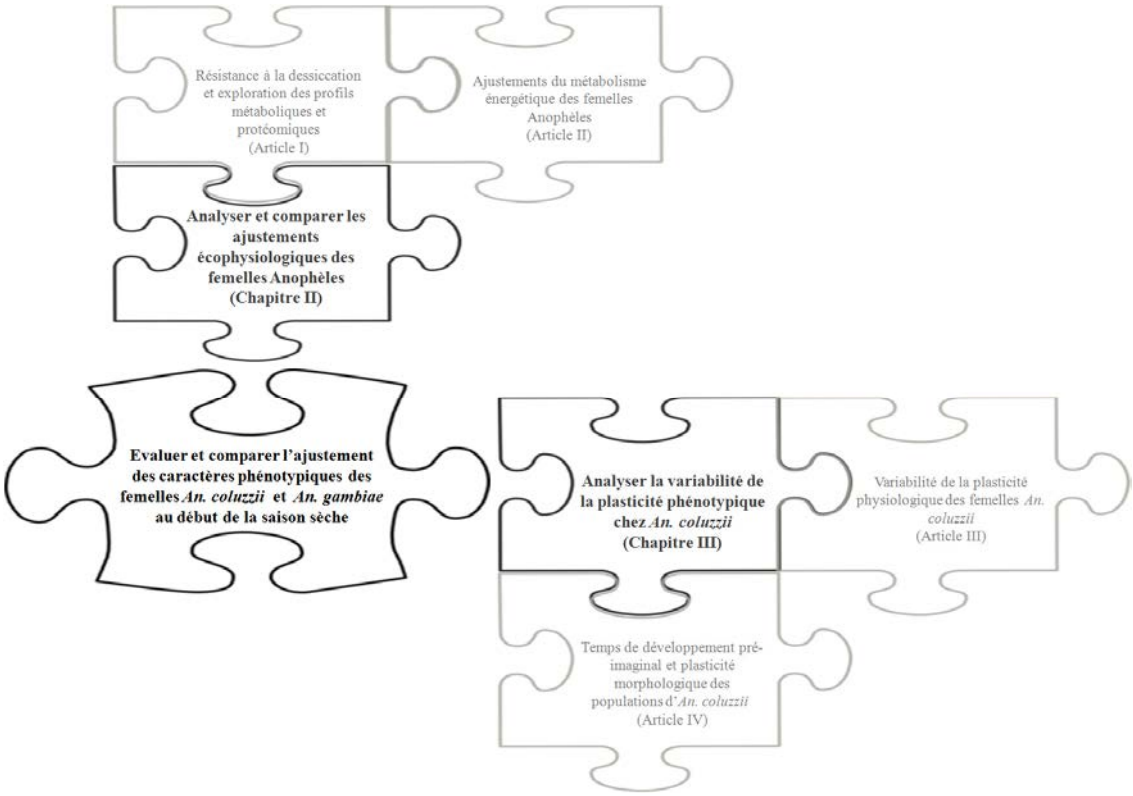


Fig. 21 Construction schématique résumant notre démarche expérimentale dans le cadre de cette thèse.

Chapitre II

Ecophysiologie comparative des réponses écophysiologiques à la saison sèche chez les Anophèles

1. **Résistance à la dessiccation et profilage métabolique et protéomique des femelles Anophèles** (Article I)

2. **Ajustements du métabolisme énergétique chez les femelles *An. coluzzii* et *An. gambiae*** (Article II)

1. Résistance à la dessiccation et profilage métabolique et protéomique des femelles Anophèles (Article I)

Les précédents travaux menés par diverses équipes de recherche suggèrent l'établissement de mécanismes de survie par les femelles *An. coluzzii* et *An. gambiae* à l'approche de la saison sèche (Adamou *et al.*, 2011 ; Huestis & Lehmann, 2014 ; Lehmann *et al.*, 2010, 2014). Ces mécanismes de survie seraient différents entre les deux espèces, ce qui suggère une variabilité de la plasticité phénotypique des femelles et des ajustements différentielles au début de la saison sèche. Cependant, ces études reposent essentiellement sur des analyses indirectes des stratégies de survie des femelles Anophèles basées sur l'étude de la dynamique et de la génétique des populations de ces espèces dans leur milieu. De plus, à notre connaissance, peu d'études ont évalué et comparé les bases écophysiologiques de ces réponses chez les femelles Anophèles (excepté Huestis *et al.*, 2011, 2012 ; Mamai *et al.*, 2014). Pourtant, l'ajustement des traits écophysiologiques des femelles exposées à des conditions reproduisant les conditions de la saison des pluies et de la saison sèche permettraient d'identifier la nature des mécanismes de survie des femelles. Dans le cadre de ce premier travail nous proposons d'explorer ces ajustements chez les femelles *An. coluzzii* et *An. gambiae*, afin d'identifier les potentiels marqueurs écophysiologiques associés aux mécanismes de survie établis par les femelles, au début de la saison sèche. Des méthodes de profilages à grande échelle du métabolome (GC-MS et UPLC) et du protéome (2D DIGE) de ces espèces ont été utilisées. Nous avons adopté une approche comparative entre les femelles *An. coluzzii* et *An. gambiae* et selon les conditions d'élevage : femelles exposées depuis le stade embryonnaire à des conditions environnementales contrastées reproduisant les conditions de la saison des pluies (RS) et du début de la saison sèche (ODS). Les dosages ont été réalisés 1h et 24h après émergence des imagos, car nous supposons que les premières heures suivant l'émergence des imagos sont critiques pour l'établissement des phénotypes, ce qui devrait se caractériser par des ajustements écophysiologiques marqués entre les deux conditions d'élevage (RS, ODS). Parallèlement, des analyses de survie et des mesures des pertes en eau corporelle des insectes ont été réalisés afin d'évaluer le

degré de résistance des femelles *An. coluzzii* et *An. gambiae* à la dessiccation. Nous supposons que les femelles exposées aux conditions du début de la saison sèche devraient alors exprimées de meilleures capacités de résistance à la dessiccation que les femelles exposées aux conditions de la saison des pluies. Ces résultats ont été mis en relation et discutés avec nos données de profilages métaboliques et protéomiques.

Les résultats obtenus ont fait l'objet d'une publication actuellement en presse dans « Journal of Insect physiology ».

Novel insights into the metabolic and biochemical underpinnings assisting dry-season survival in female malaria mosquitoes of the Anopheles gambiae complex - (Article I)

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Abstract

The mechanisms by which *Anopheles gambiae* mosquitoes survive the desiccating conditions of the dry season in Africa and are able to readily transmit malaria soon after the rains start remain largely unknown. The desiccation tolerance and resistance of female *An. gambiae* M and S reared in contrasting environmental conditions reflecting the onset of dry season ("ods") and the rainy season ("rs") was determined by monitoring their survival and body water loss in response to low relative humidity. Furthermore, we investigated the degree to which the physiology of 1-h and 24-h-old females is altered at "ods" by examining and comparing their quantitative metabotypes and proteotypes with conspecifics exposed to "rs" conditions. Results showed that distinct biochemical rearrangements occurred soon after emergence in female mosquitoes that enhance survival and limit water loss under dry conditions. In particular, three amino acids (phenylalanine, tyrosine, and valine) playing a pivotal role in cuticle permeability decreased significantly from the 1-h to 24-h-old females, regardless of the experimental conditions. However, these amino acids were present in higher amounts in 1-h-old female *An. gambiae* M reared under "ods" whereas no such seasonal difference was reported in S ones. Together with the 1.28- to 2.84-fold increased expression of cuticular proteins 70 and 117, our data suggests that cuticle composition, rigidity and permeability were adjusted at "ods". Increased expression of enzymes involved in glycogenolytic and proteolytic processes were found in both forms at "ods". Moreover, 1-h-old S forms were characterised by elevated amounts of glycogen phosphorylase, isocitrate dehydrogenase, and citrate synthase, suggesting an increase of energetic demand in these females at "ods".

Keywords: Body water; Desiccation; Metabolic fingerprint; Proteomics; RR-2 cuticular protein; Tricarboxylic acid cycle enzyme

Abbreviations: TCA= tricarboxylic acid cycle; RH= relative humidity; "rs"= rainy season; "ods"= onset of the dry season; LDA= linear discriminant analysis; LD1= first axis of the linear discriminant analysis; LD2= second axis of the linear discriminant analysis

1. INTRODUCTION

Dry savannahs of West Africa are characterised by the occurrence of two contrasting environmental seasons= a dry season, with important daily fluctuations in air temperature and significant decrease of the relative humidity, and a rainy season, during which temperature fluctuations flatten and water ponds are refilled. The alternation of these two distinct seasons has a strong impact on the life cycle of many insect species, including mosquitoes (Fontenille *et al.*, 1997; Touré *et al.*, 1998). In particular, malarial mosquito densities have been found to follow the pace of breeding-site dynamics (*i.e.*, water ponds). Hence, the pattern of malaria transmission is highly seasonal, and transmission mainly occurs during the rainy season when mosquito densities are the highest (Lemasson *et al.*, 1997).

Water availability represents an important environmental driver setting the seasonal phenology of mosquito species. Mosquitoes in the *Anopheles gambiae* complex, especially the M and S molecular forms of *An. gambiae sensu stricto*, are the major vectors of malaria in the Sub-Saharan Africa (Fontenille & Simard, 2004)*. To date, it is not known how malaria vectors survive the climatic dry-season conditions, and are able to readily achieve explosive population growth as soon as the rains start again (Lehmann *et al.*, 2010; Simard *et al.*, 2000). Of note, in areas where there are large-scale agricultural irrigation schemes or dams, and thus permanent water ponds, the M molecular form of *An. gambiae s.s.* breeds all year long, thereby extending malaria transmission into the dry season (Baldet *et al.*, 2003; Touré *et al.*, 1998). Interestingly, the genetically distinct S form of *An. gambiae* – assumed to be the ancestral form – is not found in such settings (Gimonneau *et al.*, 2012a). Population densities of the S form typically drop below the sampling detection limit soon after the onset of the dry season (Adamou *et al.*, 2011; Lehmann *et al.*, 2010), precluding field investigations of the mechanisms underlying dry-season survival strategy in this mosquito. Meanwhile, investigations of population genetics ruled out severe demographic bottlenecks in areas with strong seasonal fluctuations on the abundance of these mosquitoes. These results suggest that a significant number of adult specimens survive locally during the dry season (Simard *et al.*, 2000; Taylor *et al.*, 1993), as the pre-imaginal stages (eggs, larvae and pupae) are not able to survive

*Based on bionomical and genetic data, the two forms were recently elevated to species, and named *An. coluzzii* and *An. gambiae*, respectively (Coetzee *et al.*, 2013). Meanwhile, a consensus has not yet been reached on the species status of these incipient taxa (see Lee *et al.*, 2013), and therefore we continue to refer to the M and S nomenclature in this paper.

without water (Koenraadt *et al.*, 2003; Minakawa *et al.*, 2001). Hence, it was generally proposed that mosquito populations can be maintained in large numbers during the dry season *via* aestivating females that do not reproduce (Holstein, 1954; Lehmann *et al.*, 2010; Omer & Cloudsley-Thompson, 1970), and thus survive the challenging desiccating environmental conditions.

Desiccation represents a significant environmental stress for insects and can drive their geographical distributions (Chown & Nicolson, 2004), locomotor activities (Hoffmann & Parsons, 1993), reproduction (Benoit *et al.*, 2010a), and longevity (Hoffmann & Harshman, 1999). In insects, body water can be lost by excretion from oral and anal openings, respiratory gas exchange, and cuticular transpiration. Cuticular transpiration represents an important route of body water loss, and can account for up to 80% of the total body water loss in quiescent insects (Quinlan & Gibbs, 2006). Interestingly, body water loss is significantly minimised in insects inhabiting xeric environments= among other mechanisms, discontinuous (or cyclic) gas exchange, and decreased cuticular permeability *via* alteration of the biochemical composition are elaborated in such insects to limit body water loss (Chown *et al.*, 2011).

A primary barrier to cuticular water loss in insects is cuticular lipids, which confer most of the waterproofing in several species (Hadley, 1994). However, no direct relationship between body water loss, and the qualitative and/or quantitative nature of surface lipids of the cuticle was observed among specimens of *Drosophila* sp. (Diptera, Drosophilidae) from distinct habitats, or among specimens of *Drosophila mojavensis* acclimated to distinct thermal conditions (Gibbs *et al.*, 1998, 2003). Other than lipids, the chemical composition of the cuticle includes sugars, proteins and peptides rich in amino acids such as alanine, arginine, glycine, proline, and valine (Neville, 1975; Stankiewicz *et al.*, 1996). Among amino acids, aromatic ones, such as phenylalanine, tryptophan, and tyrosine, also represent important components of the exoskeleton. Depending on the amounts in which they are present, these aromatic amino acids can influence the hydrophobicity of the insect cuticle (Andersen, 1979). In addition, the possible involvement of cuticular proteins in water regulation has recently been reemphasised (Benoit *et al.*, 2010a), but this assumption remains to be documented conclusively. Lastly, dehydration can be tolerated by the accumulation of compatible solutes, including polyols, sugars, and amino acids (Yancey, 2005). In

particular, polyols and sugars can efficiently replace the water that surrounds membranes and proteins (see the "water-replacement hypothesis", (Crowe, 1992)).

In the present study, we explored the physiological and biochemical adjustments of freshly emerged M and S forms of *An. gambiae s.s.* in response to developmental acclimation to environmental conditions typical of the rainy ("rs") and the onset of the dry ("ods") seasons in Burkina-Faso. We assumed that developmental plasticity and acclimation play pivotal roles in augmenting the survival capabilities of mosquitoes during the dry season. We hypothesised that both molecular forms would display altered seasonal phenotypes between the dry and rainy seasons. We expected (i) distinct levels of resistance (*i.e.,* reduced body water loss) and tolerance (*i.e.*, survival probability) to desiccation between "rs"- and "ods"-reared mosquitoes in both molecular forms, (ii) physiological and biochemical rearrangements elicited by desiccation, including the accumulation of compatible solutes, changes in the expression level of structural proteins involved in the chemical composition of the cuticle, and (iii) increased expression of the proteins involved in the energetic metabolism (*i.e.,* TCA cycle) in "ods"-reared mosquitoes demonstrating the higher energetic demands (synthesis of compatible solutes, migration to more favourable microhabitats) imposed by the arrival of the dry season. We also assumed that because of the distinct phenology observed between M and S forms in the dry savannahs of West-Africa, these two molecular forms should express distinct phenotypes reflecting the different survival strategies involved by adults during the dry season. In particular, the M form should be characterised by a higher desiccation resistance than the S form, whereas signs of increased energy production supporting migratory activity should be observed in the S form. In this work, the desiccation resistance of female *An. gambiae* M and S was determined by monitoring their body water loss and survival in contrasting environmental conditions. Further, the quantitative metabotypes and proteotypes were measured in 1-h and 24-h-old adult female mosquitoes. Both metabolomics and proteomics were used to emphasise some of the biochemical adjustments leading to an alternate phenotype during the dry season in these mosquitoes.

2. MATERIALS AND METHODS

2.1. Mosquitoes

2.1.1. Source-mosquito colonies

Experiments were conducted using two *An. gambiae* colonies established from gravid M and S females collected in Burkina-Faso from human dwellings of Bama (11°23′N, 04°24′W) and Soumousso (11°01′N, 04°02′W), respectively (see Mouline *et al.*, 2012 for the entomological context of these localities). The colonies were maintained at the Institut de Recherche en Sciences de la Santé (IRSS) in Bobo-Dioulasso under controlled conditions (27 ± 1 °C, 80 ± 10% relative humidity with a 12 h=12 h L=D cycle), and routinely blood fed on restrained rabbits prior to being used for the experiments. The mosquito colonies were checked monthly using PCR methods (Santolamazza *et al.*, 2008) to ensure the absence of hybrid contamination between the M and S molecular forms of *An. gambiae*.

2.1.2. Experimental conditions

Mosquitoes were reared, from eggs to adults, in programmable climatic chambers (Sanyo MLR 315H, Sanyo Electric Co., Osaka, Japan, N = 2 climatic chambers for each experimental condition). Temperature and relative humidity (RH) cycles were programmed into the climatic chambers using climatic data hourly recorded from the village of Bama with a Vantage Pro2 weather monitoring station (Weatherlink; Davis Instruments, Hayward, CA, USA) from August 1st to 31th 2010 (*i.e.*, during the rainy season, hereafter referred to as "rs" conditions) and from December 1st to 31th 2010 (*i.e.*, at the onset of the dry season, hereafter referred to as "ods" conditions). Hourly recorded temperatures and RH from the entire months were averaged, and then a 12-step cycle was designed to reproduce as closely as possible the natural daily climatic fluctuation of "rs" and "ods" in the climatic chambers. Temperature (°C) and relative humidity (RH) were tightly monitored inside each climatic chamber using MicroLog Pro monitors (EC750, Davis Instruments, Hayward, CA, USA) (**Fig. 1**), and desiccant (Silica gel Chameleon©) was used to prevent humidity bursts in the "ods" conditions. Variation in photoperiod is greatly reduced in the tropics (*ca.,* 1-h 15 min day length

variation between the solstices in Bobo-Dioulasso, Burkina-Faso); therefore, the same photoperiod was used for the "rs" and "ods" conditions (12=12 h, L=D cycle).

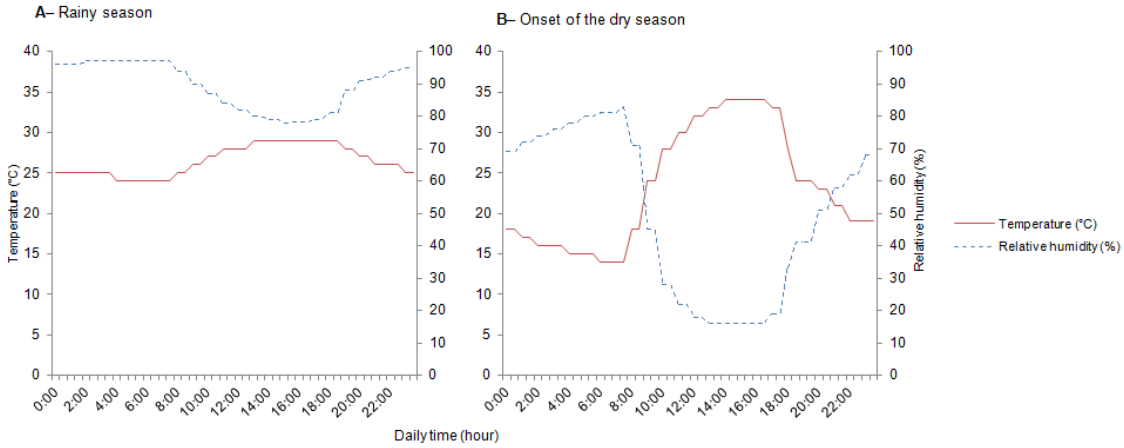

Fig. 1 Daily temperature (°C – red line) and relative humidity (% – dashed blue line) used to simulate the natural climatic variations within the climatic chambers= (A) rainy season ("rs"), and (B) onset of the dry season ("ods"). The L=D cycle was set at 12 h=12 h in both conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.1.3. Insect rearing

Mosquitoes were reared in three independent sessions from fresh batches of eggs obtained from more than 50 caged females for each molecular form. Two batches of eggs were merged to achieve large sample sizes and synchronous hatching for each session. Females in the first two sessions were used for metabolomic and proteomic assays, and females from the third batch were used for desiccation tolerance assays (*i.e.*, body water content and survival). For each environmental chamber (N = 4), the experimental conditions ("rs", "ods") were switched between the sessions.

Upon collection in the colony cages, mosquito eggs were directly transferred into plastic trays (30 cm × 20.5 cm × 6.5 cm) containing deionised water, and immediately exposed to "rs" or "ods" conditions in the climatic chambers. After hatching, first instar larvae were quickly transferred into new plastic trays at an optimal rearing density of 200 larvae per tray. Fifteen trays (*i.e.,* 3000 larvae) were used for each mosquito colony and each experimental condition ("rs", "ods"). Every day, the position of the trays was randomly alternated to avoid positional effects within the incubators. The larvae were fed daily *ad libitum* with ground fish food (Tetramin®) until pupation. Pupae were then collected and put into a plastic cup

(diameter 7 cm × height 8.5 cm) filled with 10 ml of deionised water, and covered with a fine net to prevent emerging adults from escaping. Males were discarded upon emergence, and only females were used in further experiments. The females were deprived of food throughout the experiments to control for any possible physiological difference that would have resulted from differential feeding among individuals.

Upon emergence, adult females were randomly assigned to one of four experimental treatments (1-h or 24-h exposure to the "rs" or "ods" conditions). Adult emergence was checked every hour, and newly emerged adults were immediately snap frozen, and stored at -80 °C for further metabolomic and proteomic assays (*i.e.,* 0 to 1-h-old mosquitoes, hereafter named 1-h-old mosquitoes). The remaining females were maintained in the plastic cups in the climatic chambers and exposed to "rs" or "ods" conditions for an additional 23 h period (*i.e*., 23 to 24-h-old mosquitoes, hereafter named 24-h-old mosquitoes), collected and stored for further metabolomic assays. Pools of 5–6 females from the two first rearing sessions were collected for each experimental condition for the metabolomic assays, and pools of 30 females were used for the proteomic assays.

2.2. Desiccation tolerance of female mosquitoes

2.2.1. Changes in body water content and dry mass

Fresh and dry masses were assessed using a microbalance (Sartorius SE2,*d* = 1 µg) in a subset of freshly formed pupae and 1-h-old females of both molecular forms reared under "rs" or "ods" conditions. Body water content was calculated for each female as the difference between fresh and dry mass, and expressed in mg per mg of fresh mass. The gender of the pupae was determined by morphological observation under a binocular (x40, Leica S6D), and female pupae were gently moved on an absorbent paper using a small brush to remove external water before they were weighted. Eighty-seven pupae and 140 freshly emerged females (1-h-old) were used to conduct this assay.

To examine desiccation tolerance of both molecular forms, we used 24-h-old females from both "rs" and "ods" settings. These females were provided only with cotton balls soaked with water upon emergence. This access to water was removed

after 12 h, as differential water uptake among the mosquitoes prior to the experiments would have contributed to experimental variation. Then, the 24-h-old females were put into plastic cups covered with nets. The cups were placed into large plastic boxes filled with desiccant (Silica gel Chameleon©), lowering RH inside the cup to 5–7% as measured by a T/H recorder MSR145 (MSR Electronics, GmBH, Switzerland). The boxes containing the cups were stored in a climatic chamber at 27 °C. Mosquitoes were sampled 0, 3, 6, 9, and 12 h after the females were exposed to these "low RH" experimental conditions (*i.e.*, female mosquitoes were sampled when they were 24-h, 27-h, 30-h, 33-h and 36-h old), in order to monitor changes in body water content over time. After each collection, females were quickly chloroform-anesthetised for 30 s prior to measuring their fresh mass (Sartorius SE2, d = 1 µg). Then, all specimens were dried for three days in an incubator at 60 °C, allowing the measurement of the dry mass and body water content for each female. A total of 611 female mosquitoes were used to conduct this assay.

2.2.2. Survival

Survival under desiccating conditions was assessed in females of both the M and S molecular forms reared under "rs" and "ods" environmental conditions, using previously described protocols (Fouet *et al.*, 2012; Gray *et al.*, 2009). Twenty-four hour old females were supplied with water for the first 12 h, and were individually placed into plastic vials (28.5 mm diameter × 95 mm height) filled with 3 cm of desiccant (Silica gel Chameleon©), a piece of cotton to avoid the direct contact between the mosquito and the desiccant, and sealed with a plastic film (Parafilm©). The sealed vials were stored at 27 °C into a climatic chamber. The relative humidity inside each vial dropped to 5–7% within 2–3 h from the start of the experiment. The same experimental conditions were used for the controls, except the RH which was set to 80%. In all experimental conditions, female survival was assessed hourly by observing the mosquitoes and the specimens were considered dead when they could no longer stand on their legs.

2.3. Metabolic fingerprinting

Metabolic fingerprinting was conducted on the 1-h and 24-h-old females of both M and S forms of *An. gambiae* reared under "rs" and "ods" conditions that were preserved at -80 °C. Each sample consisted of a pool of 5–6 females to achieve a minimum sample dry mass of 1 mg. For each experimental condition and for each molecular form, 5–9 replicates were used for metabolic fingerprinting, resulting in a total of 54 samples.

2.3.1. Extraction of amino acids and sugars

Mosquitoes were freeze-dried (Lyovac™ GT3) for 72 h. Dry mass was measured for each sample using a micro-balance (Mettler Toledo GmbH©, Greinfense, Switzerland, *d* = 1 µg). A 1000 µL volume of methanol-chloroform (2=1, v=v) solution was added to each sample and further homogenised with 3 mm tungsten beads at 30 Hz for 1.5 min (Retsch™ MM301, bead-beating, Retsch GbmH, Haan, Germany). A volume of 500 µL of ultrapure water was added (final methanol chloroform water solution 2=1=2, v=v=v), and the samples were further homogenised using a vortex. Samples were then centrifuged at 4000*g* for 10 min at 4 °C. A 600 µL aliquot of the upper aqueous phase, which contained amino acids and sugars, was transferred into a clean microtube and vacuum-dried (Speed Vac Concentrator, Genevac Ltd., Ipswitch, England). A volume of 600 µL of ultrapure water was added to the residual, and samples were stored at -80 °C before being used for the quantification of amino acids and sugars.

2.3.2. Circulating amino acid content

A 20 µL volume of each sample extract was diluted in 60 µL of ultrapure water, from which 5 µL were used for amino acid derivatisation according to the AccQTag ultra derivatisation kit protocol (Waters Corporation, Milford, MA, USA). A volume of 1 µL of the derivatisation mix was used for amino acid analysis. We used an Acquity UPLC® system (Waters Corporation, Milford, MA, USA) equipped with an Acquity UPLC® BEH C18 1.7 µm 2.1 × 100 mm column heated at 55 °C, as described by (Renault *et al.*, 2010). The derivatised amino acids were detected at 260 nm using a

photo diode-array detector. Peaks were identified according to their retention time compared with commercial standards, and quantified by comparison with the individual external standards of each amino acid (Renault *et al.*, 2010). The amino acid amounts were expressed in nmoles per mg of dry mass.

2.3.3. Sugar content

Sugars were quantified by gas chromatography coupled with mass spectrometry (GC–MS). A 50 µL volume of each sample extract was transferred into a glass vial and vacuum-dried. The residuals were resuspended in 50 µL of freshly prepared methoxyamine hydrochloride (Sigma–Aldrich, St. Louis, MO, USA) in pyridine (20 $mg.mL^{-1}$) prior to incubation under orbital shaking at 30 °C for 90 min. Following incubation, a 50 µL volume of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added, and derivatisation was conducted overnight at 37 °C. The GC–MS system consisted of a TriPlus autosampler, a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). The injector temperature was held at 260 °C. The oven temperature remained at 70 °C for 4 min after the injection, and then increased at a rate of 5 °C min^{-1} until it reached 300 °C, where it remained for 10 min. A 30 m fused silica column (TR5 MS, I.D. 0.25 mm, 95% dimethyl siloxane, 5% phenyl polysilphenylene-siloxane) was used with helium as the carrier gas at a rate of 1 $mL.min^{-1}$. One microlitre of each sample was injected using the split mode (25=1). MS detection was achieved using electronic impact (EI). The temperature of the ion source and MS transfer line were set to 260 °C. All of the samples were run under the SIM mode (electron energy= -70 eV). The GC–MS peaks were accurately annotated using both mass spectra (two specific ions), and a retention index specific to each compound. Randomised sample sequences were established for the sample injection. Chromatograms were deconvoluted using *XCalibur* v2.0.7. Standard samples consisting of the pure reference compounds (arabinose, fructose, galactose, glucose, ribose and trehalose) at 100, 200, 300, 500 and 1000 µM concentrations were run, and the metabolite levels were quantified using the quadratic calibration curves for each reference compound.

2.4. Proteomic assays

Proteomic assays were conducted on pools of 30 females and only 1-h-old females were used. The samples were delivered to the proteomics department of Applied Biomics (http=//www.appliedbiomics.com, Applied Biomics, Inc. Hayward, CA, USA) for processing.

2.4.1. Protein extraction procedure

For each sample, proteins were extracted using 2-D cell-lysis buffer (30 mM Tris–HCl, pH 8.8, containing 7 M urea, 2 M thiourea, and 4% 3-[(3-Cholamidopropyl) dimethylammonio]-1 propanesulfonate [CHAPS]). The protein concentration was determined using the Bio-Rad protein assay method, and the protein level was adjusted to 5 $mg.ml^{-1}$ in each sample using the 2-D cell-lysis buffer.

2.4.2. CyDye labelling and two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)

CyDye DIGE Fluor Cy3 and Cy5 saturation dyes (4 mL, 2 mM) were used to label the "ods" and "rs" samples, respectively. A third dye, Cy2, was used to label a 50=50 mixture of the two samples, which became the internal standard for the normalisation of spot abundances. The samples were incubated with cyanine dyes for 30 min on ice under dark conditions before adding a 1 µL volume of 10 mM lysine. The reaction was stopped by the addition of 2× 2-D sample buffer (8 M urea, 4% CHAPS, 20 $mg.mL^{-1}$ DTT, 2% pharmalytes and a trace amount of bromophenol blue), and the samples were stored at -80 °C until the 2D-DIGE experiment. Just prior to 2D-DIGE, equal amounts of Cy3- and Cy5-labelled samples (30 µg each) were mixed with rehydration buffer. After adding DeStreak™ rehydration solution (GE Healthcare, Waukesha, WI, USA) containing 0.5% pharmalyte pH 3–10 in a 250 µL final volume, the samples were loaded onto an isoelectric focusing (IEF) strip (pH 4–10 linear range; GE Healthcare). The IEF was conducted with standard conditions using Ettan IPGPhore II. After the IEF, the strips were incubated in equilibration buffer (50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue and 10 $mg.mL^{-1}$ DTT) for 15 min. The strips were then rinsed in

equilibration buffer 2 (50 mM Tris–HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, a trace amount of bromophenol blue and 45 $mg.mL^{-1}$ iodoacetamide) for 10 min. Then, electrophoresis was performed at 15 °C on a 12% SDS–PAGE gel.

Each gel contained three samples pre-labelled with different dyes= (i) the "ods" sample (labelled with Cy3), (ii) the "rs" sample (labelled with Cy5) and (iii) the 50=50 mixture of the two samples used to normalise the spot abundance (labelled with Cy2). For each molecular form (*An. gambiae* M and *An. gambiae* S), three different replicate gels were run to compare the effects of the experimental conditions ("ods", "rs") on protein expression, and to allow subsequent statistical assessments ($N = 6$ gels in total). Finally, a seventh gel containing both of the molecular forms from the "ods" conditions and their mixture was run. Comparisons between *An. gambiae* M and S were conducted by overlaying images of the gels and the abundance of each spot was normalised using the results gathered from the seventh gel. The resulting 2D gels were scanned using a Typhoon Trio scanner (Amersham BioSciences) with excitation and emission wavelengths for Cy2-labelled (488/520 nm), Cy3-labelled (548/560 nm) and Cy5-labelled (641/660 nm) proteins using settings that resulted in similar relative fluorescence intensities for the Cy3- or Cy5-labelled samples. Image analysis for intensity measurements of the protein spots chosen was performed using the ImageQuantTL and DeCyder software (GE Healthcare), and then, the images were subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE-Healthcare) with a P-value <0.05 (Student's t-test). The ratio of the different protein expression between the species and/or conditions was obtained from in-gel DeCyder software analysis (see gel representations in **Supplementary data 1**).

2.4.3. Protein identification using matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) and tandem mass spectrometry (MS)

Protein identification was performed by Applied Biomics, Inc. After analyses of the 2D-DIGE image, selected protein spots of interest (see the data analysis section) were picked up from the gel using an Ettan spot picker (GE Healthcare). The gel spots were washed twice with 25 mM ammonium bicarbonate and 50% acetonitrile to remove the staining dye. The proteins were digested in-gel at 37 °C with modified porcine trypsin

protease (Trypsin Gold, Promega) (Rosenfeld *et al.*, 1992). The digested tryptic peptides were desalted using Zip-tip C18 (Millipore, Billerica, MA), mixed with 0.5 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix and spotted into wells of a MALDI plate. Mass spectra of the peptides in each digested spot were obtained using MALDI-TOF (MS) and TOF/TOF (tandem MS/MS) equipment (AB Sciex). The MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 2000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 2000 laser shots per fragmentation spectrum on each of the 5–10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions). Both the resulting peptide mass and the associated fragmentation spectra were submitted for database search using GPS Explorer software version 3.5 (Applied Biosystems) equipped with the MASCOT search engine (http=//www.matrixscience.com, Matrix science) to identify proteins from the National Center for Biotechnology Information non-redundant protein sequence database (NCBInr), restricted to *An. gambiae*. The searches were performed without constraining the protein molecular weight or isoelectric point, with carbamidomethylation and oxidation as variable modifications, and with one missed cleavage allowed in the search parameters. The highest protein scoring hit with a protein score confidence interval over 95% from the database search for each 2D gel spot was accepted as positive identification.

2.5. Data analysis

All statistical procedures were conducted with the *R* 2.15.0 statistical software (R Development Core Team, 2008). Before analysis, the normal data distribution and homoscedasticity of each dataset were determined using Shapiro–Wilk and Bartlett's tests, respectively. Accordingly, parametric or non-parametric tests were further processed. Analyses of covariance (ANCOVA) were performed to address the effects of the molecular form, experimental conditions, and female age on the body water content of female mosquitoes during development (*i.e.*, pupae to 24-h-old) and under exposure to 5–7% RH of "rs" and "ods" acclimated mosquitoes. Dry mass was used as a covariable in these models. The effect of molecular form, experimental conditions,

and female's age on female's dry mass was assessed using a three-way ANOVA. Model simplification used stepwise removal of terms followed by the estimate of Akaike information Criterion (AIC), as described by (Crawley, 2007). Term removals that significantly reduced explanatory power were retained in the minimal adequate model. Tukey's *post hoc* procedures were used to perform comparisons among the levels of significant factors.

Further, non-parametric regression analyses (Cox proportional hazard models) were performed to examine the effect of molecular forms (M, S), and experimental conditions ("rs, "ods") on the survival of the 24-h-old females submitted to both desiccating (5–7% RH) and control conditions (80% RH). For these analyses, the time to death was defined as the response variable, and the mosquito molecular form, dry mass, experimental condition ("rs" *vs.* "ods"), and exposure to desiccating conditions in the plastic vial (5–7% *vs.* 80%) were considered as the explanatory variables. Model simplification used stepwise removal of terms, where the significance of the terms was estimated using the difference in Akaike's information criterion (AIC). As all mosquitoes were dead at the end of this experiment, no survival time was censored. Survival distributions were illustrated by Kaplan–Meier survivorship curves.

Multivariate discriminant analyses were used to assess differences in the amount of amino acids among molecular forms, experimental conditions, and females' age (*i.e.*, 1-h and 24-h-old mosquitoes). The amino acid concentrations were log-transformed ($x = \log_{10}[X + 1]$) to fulfil the assumption of normally distributed residuals. We first performed MANOVAs to address differences in amino acid amounts between the two molecular forms of *An. gambiae*, the two experimental conditions, and across the females' age classes. Samples for which some of the amino acids were not reliably quantified (Signal/Noise < 10, or concentration < Quantification Limit) were discarded from the analysis. Class separation was further investigated using linear discriminant analyses (LDA). Taking into account the significant effect of the molecular form on the amino acid signatures, LDA were performed separately for *An. gambiae* S and M. The significance distribution of each LDA was assessed using Monte Carlo 10,000 permutations ($P < 0.001$). Two-way ANOVAs (where the experimental conditions and females' age were considered as

continuous variables) were conducted in parallel for each individual metabolite and for each molecular form. These analyses were followed, when necessary, by Tukey *post hoc* tests among levels of significant factors. The between- and within-groups degrees of freedom together with the *F*-value are reported. The *P*-values were adjusted using the Benjamini Hochberg algorithm (Benjamini & Hochberg, 1995) to control the false discovery rate induced by multiple comparisons ($P < 0.05$). Metabolites for which no significant difference was observed for at least one variable were discarded from the discriminant analysis to accurately explain the physiological differences among the groups. For the sugar amounts, non-parametric Kruskal–Wallis tests were conducted to examine possible differences among the two molecular forms of *An. gambiae*, the two experimental conditions, and the two female age classes (1-h- and 24-h-old). These tests were followed by a Bonferroni correction resulting in a decrease threshold of significance to $P < 0.01$.

Finally, for average-fold differences in protein abundance, protein quantities from each 2D-DIGE gel were normalised (Log abundance). The averaged-fold differences in spot abundance were then calculated for the following ratios= (i) *An. gambiae* M "rs"/*An. gambiae* M "ods"; (ii) *An. gambiae* S "rs"/*An gambiae* S "ods"; (iii) *An. gambiae* M "rs"/*An gambiae* S "rs"; and (iv) *An. gambiae* M "ods"/*An gambiae* S "ods". For each ratio, differences in protein expression were assessed using Student's *t*-tests on individual proteins ($P < 0.05$).

3. RESULTS

3.1. Changes in body water content and dry mass

The fresh and dry masses of 909 mosquitoes were measured to determine their body water content. Overall, the water content of both M and S forms reared under "rs" or "ods" conditions decreased from pupae to 24-h-old mosquitoes (ANCOVA, $ddl = 2$, $F = 171.33$, $P < 0.001$; **Fig. 2A**), and from 0 to 12-h exposure to low (5–7%) RH (ANCOVA, $ddl = 4$, $F = 138.22$, $P < 0.001$; **Fig. 2B**). Significant variations of body water contents were observed between molecular forms and experimental conditions from pupae to 24-h-old mosquitoes (ANCOVA, $ddl = 1$, $F = 9.80$, $P < 0.01$; $F =$

24.79, $P < 0.001$, respectively). Although the M form exhibited higher amounts of body water when the females were reared under "ods" conditions, no such a difference was observed in the S form from pupae to 24-h-old females (**Fig. 2A**). In addition, acclimation to "rs" or "ods" conditions had a significant effect on the body water content of females exposed to low RH (5–7%) (ANCOVA, *ddl* = 1, $F = 195.03$, $P < 0.001$), and this effect is distinct according the molecular form (ANCOVA, *ddl* = 3, $F = 4.51$, $P < 0.01$). Accordingly, "ods" specimens had significantly higher water content than "rs" ones in M molecular forms, but no such a significant difference was observed in the S forms (**Fig. 2B**). Dry mass of both M and S females significantly affect the body water contents of female's mosquitoes from pupae to 24-h-old mosquitoes (ANCOVA, *ddl* = 1, $F = 239.90$, $P < 0.001$), and from 0 to 12-h exposure to 5–7% RH (ANCOVA *ddl* = 1, $F = 416.44$, $P < 0.001$). An increase in dry mass correlated with a decrease in body water content (**Figs. 2 and 3**). No change in dry mass was observed from 3-h to 12-h exposure to 5–7% RH conditions in both M and S forms (**Fig. 3B**).

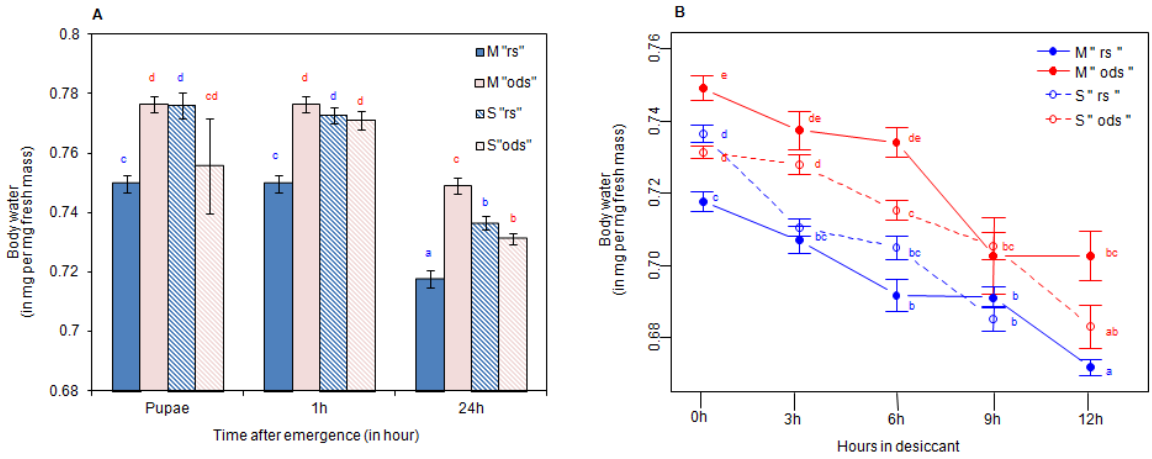

Fig. 2 Mean (±s.e.) body water content (in mg water per mg fresh mass) in *An. gambiae* M (solid bars and lines) and S (dashed bars and lines) forms reared under "rs" (blue bars and lines), and "ods" conditions (red bars and lines). Panel A shows body water content variation from pupae to 24-h-old females exposed to "rs" or "ods" conditions. Panel B reflects body water content variations from 24-h-old to 36-h-old females acclimated to "rs" or "ods" conditions and exposed to low RH (5–7%). Letters above the bars report significant differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

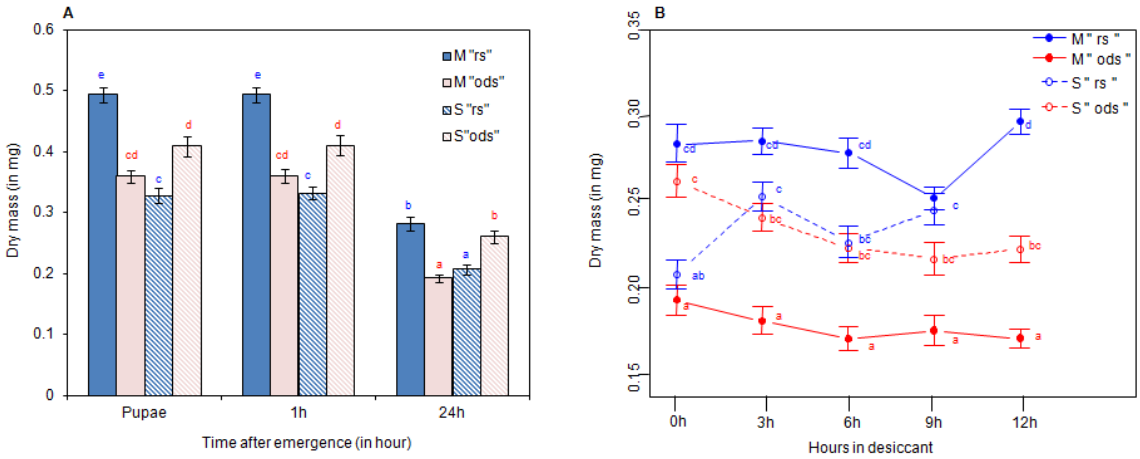

Fig. 3 Mean (±s.e.) female's dry mass (in mg) in *An. gambiae* M (solid bars and lines) and S (dashed bars and lines) forms reared under "rs" (blue bars and lines), and "ods" conditions (red bars and lines). Panel A shows dry mass variation from pupae to 24-h-old females exposed to "rs" or "ods" conditions. Panel B reflects dry mass variations from 24-h-old to 36-h-old females acclimated to "rs" or "ods" conditions and exposed to low RH (5–7%). Letters above the bars report significant differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Survival

Overall, control females exposed to high RH (80%) survived longer than those exposed to low RH (5-7%) conditions (LRT χ^2_1= 182.54, $P < 0.001$; **Fig. 4A-B**) and body mass positively affected survival duration (LRT χ^2_1 = 121.36, $P < 0.0001$). In both control (**Fig. 4A**) and low RH conditions (**Fig. 4B**), the experimental conditions ("rs", "ods") significantly influenced mosquitoes' survival (LRT χ^2_1= 60.41, $P <$ 0.001)= females survived longer when they had been reared under "ods" as compared to "rs" conditions. This was particularly true for mosquitoes exposed to low RH conditions (LRT χ^2_1= 10.10, $P < 0.001$; **Fig. 4B**).

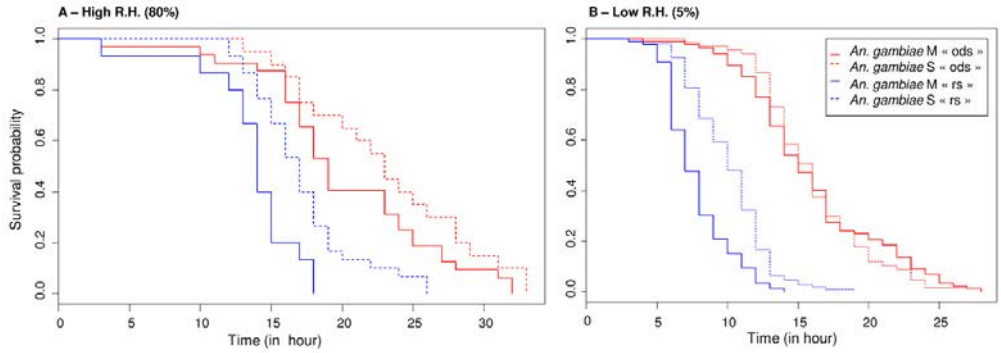

Fig. 4 Kaplan–Meier survival probability curves for 24-h-old female *An. gambiae* M (solid lines) and S (dotted lines) reared under "rs" (blue) and "ods" (red) conditions. Panel A= control high RH conditions (80%); panel B= exposure to low RH (5–7%). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Metabolic fingerprinting

3.3.1. Circulating amino acid content

Of the 22 amino acids listed in our external standard, 21 were detected and quantified. In particular, we were unable to provide reliable measurements of cysteine amounts in our samples, and this amino acid was therefore discarded from further analysis. Similarly, for technical reasons, some amino acids were not reliably quantified in some samples (signal/noise < 10, or concentration < quantification limit). Thus, four samples were discarded from the analysis to successfully achieve the LDA, including three samples of 1-h-old specimens of the M form (two reared under "rs", and one under "ods"), and one sample of 24-h-old female of *An. gambiae* S reared under "rs" conditions.

Significant differences in the concentration of at least one amino acid were found between the two molecular forms of *An. gambiae*, between 1-h and 24-h-old females, and between the two experimental conditions ("rs", "ods"; **Table 1**). The amino acid content of the two molecular forms differed according to the seasonal conditions and females' age, as demonstrated by the significance of the interaction terms (**Table 1**).

Table 1 Results of the MANOVA computed on the amino acid contents of individual females exposed to the different experimental conditions. Numeric data are rounded to two decimal digits. The Pillai trace was multiplied by 10.$^{***}P < 0.0001$; $^{**}P < 0.05$; "ns"= $P > 0.05$.

Dataset	Effects	*ddl*	Test stat	F- value	Num *Df*	*P*-value
(*N* = 54 samples)	Experimental condition	1	87.65	5.74	21	***
	Molecular form	1	82.36	3.78	21	**
	Females' age	1	91.91	9.20	21	***
	Molecular form : Experimental condition	1	82.31	3.76	21	**
	Females' age : Experimental condition	1	90.80	7.98	21	***
	Molecular form : Females' age	1	81.83	3.64	21	**
	Molecular form : Female' age : Experimental condition	1	68.59	1.76	21	ns

3.3.1.1. Effects of the environmental conditions and females' age on the circulating amino acid composition of An. gambiae *M*

The amino acid composition of female *An. gambiae* M differed significantly between the two experimental conditions (MANOVA, $F_{1,10} = 5.30$, $P < 0.01$), and between 1-h and 24-h-old females (MANOVA, $F_{1,10} = 15.76$, $P < 0.001$). Amino acid profiles were differentially altered in 1-h and 24-h-old females exposed to "rs" and

"ods" conditions, as demonstrated by the significance of the interaction term (MANOVA, $F_{1,10} = 4.18$, $P < 0.01$).

Ten out of 21 amino acids showed significant differences among experimental conditions, and were further considered for the LDA (**Supplementary data 1**). The 11 remaining amino acids (*i.e.*, alanine, aspartate, GABA, glycine, isoleucine, leucine, methionine, ornithine, proline, threonine, and tryptophan) were discarded from subsequent analysis. The first axis (LD1) accounted for 40.8% of the total inertia, and the variation among groups was 9.98 times higher than the variation within groups (**Fig. 5A**). LD1 was mainly supported by the variations of the 10 significant amino acids (mainly arginine, glutamine, histidine, phenylalanine, tyrosine, and valine) which showed higher amounts in 1-h-old females, and more particularly in females reared under "ods" conditions (**Supplementary data 2 and 3**). The second axis (LD2) accounted for 34.9% of the total inertia and the variation among groups was 5.41 times higher than the variation within groups. This axis represented a clear cut-off between females reared under "rs" and "ods" experimental conditions. This axis was mainly supported by the variation in histidine content, whose amounts were higher in 1-h-old females reared under "ods" conditions (**Supplementary data 2 and 3**).

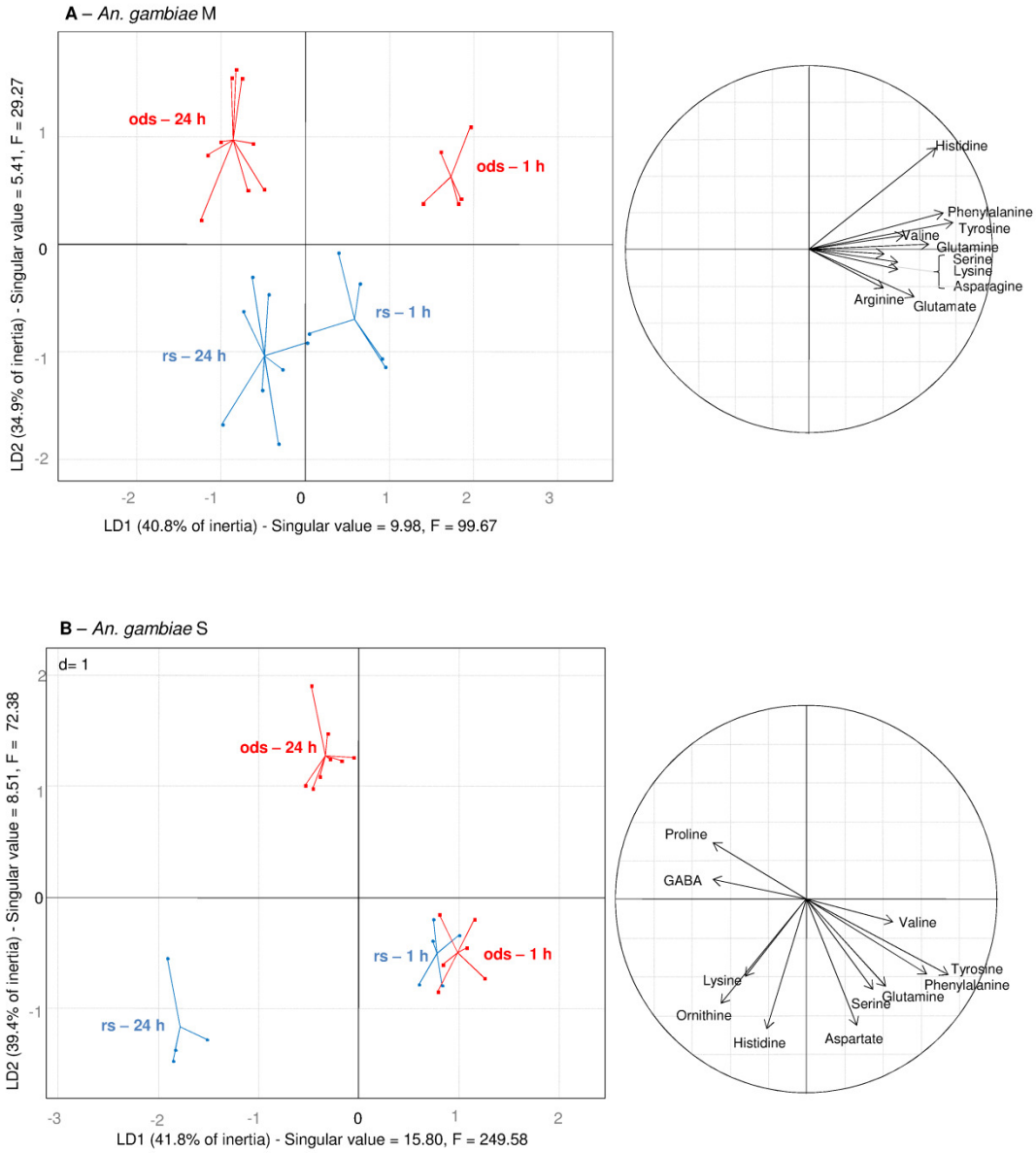

Fig. 5 Left= Sample projections onto the first LDA discriminant plane for female *Anopheles gambiae* M (28 samples, A) and S (23 samples, B) reared under "ods" (red squares) and "rs" (blue dots) conditions. Singular values refer to the ratio of the between-class and within-class inertia. Right= correlation circles depict the normalized relation (from −1 to 1) between each amino acid and LDA axes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3.1.2. Effects of environmental conditions and females' age on the circulating amino acid composition of An. gambiae *S*

The amino acid composition of *An. gambiae* S females differed significantly between experimental conditions (MANOVA, $F_{1,11} = 7.17$, $P < 0.01$), and between 1-h and 24-h-old females (MANOVA, $F_{1,11} = 28.19$, $P < 0.001$). Amino acid profiles were

differentially altered in 1-h and 24-h-old females exposed to "rs" and "ods" conditions, as demonstrated by the significance of the interaction term (MANOVA, $F_{1,11} = 6.85$, $P < 0.01$).

A LDA was performed to further analyse the differences found among the experimental groups (**Fig. 5B**). Of the 21 amino acids considered in this study, 11 showed significant differences among groups, and were therefore considered for the LDA (**Supplementary data 4**). To gain insights into the separation among groups, the 10 amino acids showing a constant concentration regardless of the experimental conditions (*i.e.*, alanine, arginine, asparagine, glutamate, glycine, isoleucine, leucine, methionine, threonine, and tryptophan) were discarded from subsequent analysis.

The first axis (LD1) accounted for 41.8% of the total inertia, and the variation among groups was 15.8 times higher than the variation within groups. LD1 corresponded to a clear cut-off between 1-h and 24-h-old females, and was mainly supported by variations in the contents of GABA, ornithine, phenylalanine, proline, tyrosine, and valine. Amino acid composition of 1-h-old females from "rs" and "ods" conditions overlapped and these groups were notably characterised by accumulation of phenylalanine, tyrosine, and valine (**Supplementary data 3 and 4**).

The second axis (LD2) accounted for 39.4% of the total inertia and the variation among groups was 8.5 times higher than the variation within groups. This axis was mainly supported by variations in the contents of several amino acids, of which aspartate, histidine, and ornithine can be mentioned (**Supplementary data 3**). LD2 corresponded to a contrast between 24-h-old females exposed to "ods" conditions and those exposed to "rs" conditions, with low levels of aspartate, histidine, and ornithine in the 24-h-old females exposed to "ods" conditions.

3.3.2. Sugar content

Of the six sugars contained in our reference standard, only two (*i.e.*, galactose and glucose) were reliably quantified in the mosquito samples; the other four (*i.e.*, arabinose, fructose, ribose, and trehalose) were below the quantification limit of our equipment. Both the galactose and glucose amounts were significantly higher in the 24-h-old M and S females exposed to the "ods" conditions (Kruskal–Wallis test, $P <$

0.01) (**Fig. 6A-D**). The glucose and galactose amounts did not differ in the 1-h-old females, except for females of the M form, whose glucose amounts decreased under the "ods" conditions (**Fig. 6C**).

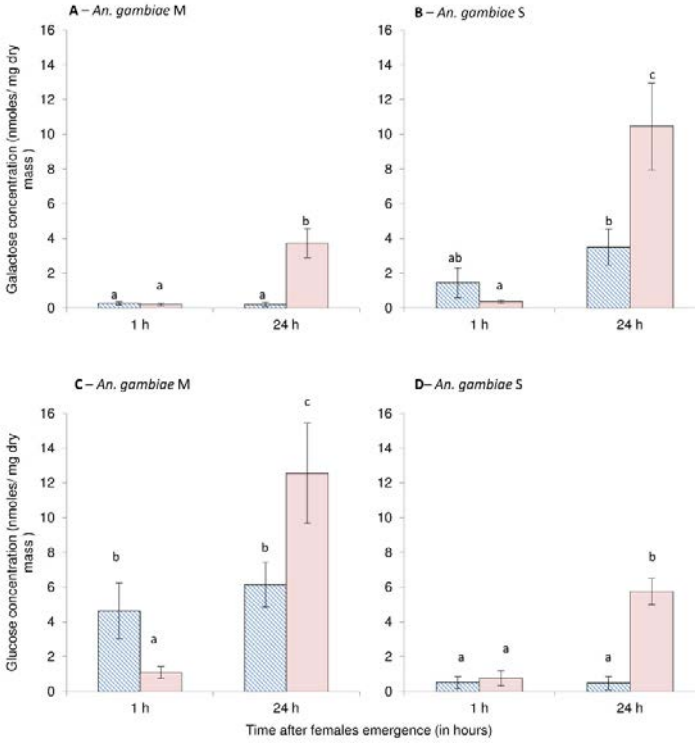

Fig. 6 Mean (±s.e.) galactose (A and B) and glucose (C and D) levels (in nmole.mg^{-1} dry mass) in 1-h and 24-h-old females of *An. gambiae* M (A and C) and S (B and D). Dashed blue bars correspond to the females reared under "rs" conditions and solid red bars correspond to females reared under "ods" conditions. Letters above the bars report significant differences after Bonferroni correction to account for multiple tests ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Proteomic assays

3.4.1. Protein content in emerging females

A total of 109 spots, corresponding to proteins with molecular masses ranging from 14 to 150 kDa, and isoelectric points between 4 and 9, exhibited significant variations in at least one molecular form and/or experimental condition. Based on an average difference of at least 1.3-fold (absolute value), 41 spots were selected, which corresponded to 33 distinct proteins.

3.4.2. *An. gambiae* M form

Fifteen proteins exhibited significant variation between the "rs" and "ods" conditions in the 1-h-old females of *An. gambiae* M, of which 10 were more abundant when the females were grown under the "ods" conditions (**Table 2, Supplementary data 5 and 6**). This group included four proteins engaged in striated muscle contraction (spots #1,

#37–39, #45, #57–60), a proteolysis enzyme (spots #24–25), a hexamerin (spot #2), an arylphorin (spot #3), a catalase (spot #19), an enzyme involved in DNA translation (spot #55), and a NADH dehydrogenase (spot #56; **Table 2, Supplementary data 7**). A RR-2 cuticular protein 70 (spot #67) was also significantly more abundant under the "ods" conditions (Student *t*-test, $P < 0.001$) although its fold change was only 1.28. Five proteins were found in lower amounts when the females were exposed to the "ods" conditions (**Table 2, Supplementary data 5 and 6),** including three storage proteins (spots #9, 12 and 21), an oxygen transporter (spot #64), and an ATP synthase *beta* subunit (spot #67).

3.4.3. *An. gambiae* S form

Fourteen proteins exhibited significant variations between the "rs" and "ods" conditions in the 1-h-old females of *An. gambiae* S, of which 8 proteins were more abundant in the females exposed to the "ods" conditions (**Table 2, Supplementary data 5 and 6**). This group included two constituents of the rigid cuticle (RR-2 cuticular protein 70 and 117, spots #67 and #65, respectively), four proteins involved in striated muscle contraction (spots #1, #37–39, #45, #57–60), two enzymes involved in metabolic pathways (*i.e.*, aspartate ammonia lyase and NADH dehydrogenase= spots #27, 56; **Table 2, Supplementary data 5**). The amounts of six other proteins were reduced when the 1-h-old females were exposed to the "ods" conditions (**Table 2, Supplementary data 5 and 6**). These proteins included three storage proteins (spots #7, 9, 12, 21, 68), an oxygen transporter (spot #64), a protein involved in motor activity (spot #13), and an enzyme involved in proteolysis processes (spot #25).

3.4.4. Difference in protein composition between the two molecular forms

Nine proteins exhibited the same pattern in the two molecular forms= five proteins were more abundant in females grown under the "ods" conditions, whereas the levels of four others decreased under these conditions (**Table 3**). A total of 24 proteins showed significant variations between the females of *An. gambiae* M and S exposed to the "rs" or "ods" conditions (**Table 3, Supplementary data 6**). Six proteins were more abundant in females of *An. gambiae* S, whatever the experimental conditions

("rs" or "ods"), among which prophenoloxidase (spot #76), citrate synthase (spot #84), isocitrate dehydrogenase (spot #85), and glutathione transferase (spot #99) can be mentioned (**Table 3, Supplementary data 7**). The RR-2 cuticular protein 123 (spot #101) was also significantly more abundant under the "ods" conditions (Student *t*-test, $P < 0.001$), although its fold change was only 1.27. In addition, a phosphorylase (spot #75) was more abundant in *An. gambiae* S compared to *An. gambiae* M when the females were exposed to the "ods" conditions, but no differential expression was found in the "rs" conditions. Arginine kinase (spot #96) was always more abundant in *An. gambiae*M, whatever the experimental conditions.

4. DISCUSSION

In the present work, we provide novel insights into the physiological and biochemical changes in freshly emerged (1-h and 24-h-old) mosquitoes *An. gambiae s.s.* that accompany acclimation to contrasting environmental conditions they experienced during the onset of the dry season in the Burkina-Faso. Our data demonstrates that developmental acclimation to dry season conditions enhances the desiccation tolerance and resistance (*i.e.*, increases survival and reduces body water loss) of mosquitoes exposed to low 5–7% RH conditions in both M and S forms. We observed significant metabolic and biochemical rearrangements elicited by exposure of immature stages to dry conditions in both molecular forms. These changes include increased amounts of circulating sugars when the mosquitoes were reared under "ods" conditions, changes in both circulating amino acid fingerprints and protein expression supporting adjustments in the cuticle composition of females of *An. gambiae* M (*e.g.*, increased amounts of tryptophane, tyrosine, valine, and RR2 cuticular proteins), and higher expression of enzymes involved in intermediary metabolism in *An. gambiae* S (*e.g.*, increased amounts of aspartate ammonia lyase citrate synthase, isocitrate dehydrogenase, and proline), suggesting higher metabolic activity in this latter species.

Table 2 Protein spots displaying differential abundance (average fold difference >1.3, absolute value; $P < 0.05$) between the "rs" and "ods" experimental conditions in 1-h-old female *An. gambiae* M (orange) and S (green). Black numbers represent protein ratios that did not significantly vary between the two experimental conditions.

Spot no.[a]	Protein name	Protein scores[b]	No. Of matched peptides	Source species	Accession no.[c]	*An. gambiae* M		*An. gambiae* S		Functions[e]
						Ratio (rs/ods)[d]	*P-value*[e]	Ratio (rs/ods)[d]	*P-value*[e]	
01	tropomyosin invertebrate	318	12	*Culex quinquelasciatus*	gi|170056897	-1.64	< 0.001	-1.83	< 0.001	Muscle contraction
02	hexamerin 2 beta	41	2	*Aedes aegypti*	gi|157119837	-1.78	< 0.001	1.01	0.51	Storage of amino acid
03	arylphorin subunit alpha	134	4	*Culex quinquelasciatus*	gi|170043201	-1.81	< 0.001	1.13	< 0.01	Storage of amino acid / Constituent of Cuticle sclerotizing system
07	hexamerin A	284	15	*Anopheles melas*	gi|3420171	1.32	< 0.01	1.51	< 0.01	Storage of amino acid
09	hypothetical protein AND_22551	153	15	*Anopheles daringi*	gi|312371166	1.22	< 0.05	1.65	< 0.01	Storage of amino acid
12	hexamerin 2 beta	70	4	*Aedes aegypti*	gi|157110143	1.88	< 0.001	2.8	< 0.001	Storage of amino acid
13	myosin-Id	172	5	*Culex quinquelasciatus*	gi|170029188	1.02	0.74	1.46	< 0.001	Muscle contraction
19	catalase	215	16	*Anopheles gambiae*	gi|118838436	-1.9	< 0.01	-1.31	0.13	Response to oxidative stresses
21	hypothetical protein AND_22551	321	12	*Anopheles daringi*	gi|312371166	1.32	< 0.01	2.06	< 0.001	Storage of amino acid
24	AGAP005558-PA	1000	19	*Anopheles gambiae*	gi|31213235	-2.05	< 0.001	NA	NA	Proteolysis
25	AGAP005558-PA	570	16	*Anopheles gambiae*	gi|31213235	-2.85	< 0.001	1.56	< 0.001	Proteolysis
27	aspartate ammonia lyase	301	15	*Aedes aegypti*	gi|157118058	1.13	< 0.05	-1.46	< 0.01	Tricarboxylic acid cycle
37	myosin regulatory light chain 2 (mlc-2)	336	5	*Aedes aegypti*	gi|157167683	-1.92	< 0.001	-2.05	< 0.001	Muscle contraction
38	myosin regulatory light chain 2 (mlc-2)	299	5	*Aedes aegypti*	gi|157167683	-1.74	< 0.001	-1.81	< 0.001	Muscle contraction
39	myosin regulatory light chain 2 (mlc-2)	330	5	*Aedes aegypti*	gi|157167683	-1.64	< 0.001	-1.73	< 0.001	Muscle contraction
45	AGAP007963-PA	601	16	*Anopheles gambiae*	gi|118789564	-1.41	< 0.001	-1.63	< 0.001	Muscle contraction
52	ATP synthase beta subunit	250	12	*Culex quinquelasciatus*	gi|170040305	2.15	< 0.01	1.2	0.22	ATP synthesis
55	AGAP011284-PA	304	11	*Anopheles gambiae*	gi|118779554	-1.43	< 0.001	-1.08	0.16	Positive regulation of translational elongation and termination
56	NADH dehydrogenase	268	6	*Culex quinquelasciatus*	gi|170037145	-1.41	< 0.001	-1.52	< 0.001	Respiratory chain process
57	myosin light chain 1, putative	202	4	*Aedes aegypti*	gi|157167807	-1.89	< 0.01	-1.54	< 0.001	Muscle contraction
58	myosin light chain 1, putative	117	4	*Aedes aegypti*	gi|157167807	-1.93	< 0.001	-1.68	< 0.01	Muscle contraction
59	myosin light chain 1, putative	156	5	*Aedes aegypti*	gi|157167807	-3.28	< 0.001	-2.25	< 0.01	Muscle contraction
60	myosin light chain 1, putative	181	5	*Aedes aegypti*	gi|157167807	-2.46	< 0.01	-2.27	< 0.01	Muscle contraction
64	AGAP010657-PA	165	8	*Anopheles gambiae* str. PEST	gi|158289706	1.84	< 0.001	2.07	< 0.001	Oxygen transporter activity
65	"cuticular protein 117, RR-2 family (AGAP003379-PA)	709	9	*Anopheles gambiae*	gi|158290052	-1.37	0.18	-2.84	< 0.001	Structural constituent of the rigid cuticle
67	"cuticular protein 70, RR-2 family (AGAP006283-PB)	808	9	*Anopheles gambiae*	gi|158295676	-1.28	< 0.001	-1.41	< 0.001	Structural constituent of the rigid cuticle
68	hexamerin A	395	26	*Anopheles gambiae*	gi|3420159	1.43	0.26	2.6	< 0.05	Storage of amino acid

[a] Protein scores derived from Mascot algorithm, indicating identity or extensive homology ($P < 0.05$)
[b] Protein accession numbers from the National Center for Biotechnology Information non-redundant (NCBInr) database
[c] Positive values mean more expressed in "rs" (rainy season) conditions whereas negative values mean over expressed in "ods" (onset of the dry season) conditions
[d] Statistics were performed using t-test, and significance was defined as $P < 0.05$.
[e] Protein functions are checked using http://www.uniprot.org

Spots 09 and 21 correspond to the same protein.
Spots 24 and 25 correspond to the same protein.
Spots 37-39 correspond to the same protein.
Spots 57-60 correspond to the same protein.
* So far, the large majority of cuticular protein sequences that have been studied in arthropods have a conserved region calls the Rebers and Riddiford Consensus (R&R Consensus).

Table 3 Protein spots displaying differential abundance (average fold difference >1.3, absolute value; $P < 0.05$) between 1-h-old female *An. gambiae* M and S reared under the "ods" (red) and "rs" (blue) conditions, respectively. Black numbers represent protein ratios that did not significantly vary between the two experimental conditions. In grey, protein spots already shown in **Table 2**.

Spot no.	Protein name	Protein scores[a]	No. Of matched peptides	Source species	Accession no.[b]	ods conditions		rs conditions		Functions[e]
						Ratio (M/S)[c]	*P-value*[d]	Ratio (M/S)[d]	*P-value*[d]	
01	tropomyosin invertebrate	318	12	*Culex quinquefasciatus*	gi|170056997	-1.62	< 0.05	-1.37	< 0.001	Muscle contraction
02	hexamerin 2 beta	41	2	*Aedes aegypti*	gi|157119837	2.12	< 0.001	1.22	< 0.01	Storage of amino acid
03	arylphorin subunit alpha	104	4	*Culex quinquefasciatus*	gi|170043201	1.36	< 0.05	-1.93	< 0.001	Storage of amino acid / Constituent of cuticle sclerotizing system
07	hexamerin A	264	15	*Anopheles melas*	gi|3420171	1.85	< 0.001	1.71	< 0.001	Storage of amino acid
09	hypothetical protein AND_22551	153	15	*Anopheles darlingi*	gi|312371166	2.57	< 0.001	1.52	< 0.001	Storage of amino acid
12	hexamerin 2 beta	70	4	*Aedes aegypti*	gi|157110143	2.31	< 0.001	1.39	< 0.001	Storage protein
13	myosin Id	172	5	*Culex quinquefasciatus*	gi|170029188	1.36	0.16	-1.85	< 0.01	Muscle contraction
19	catalase	215	16	*Anopheles gambiae*	gi|118638436	1.36	0.61	-1.37	< 0.05	Response to oxidative stresses
21	hypothetical protein AND_22551	351	12	*Anopheles darlingi*	gi|312371166	2.37	< 0.001	1.33	< 0.001	Storage of amino acid
25	AGAP005258-PA	570	16	*Anopheles gambiae* str. PEST	gi|312135235	3.42	< 0.001	-1.31	< 0.05	Proteolysis
27	aspartate ammonia lyase	301	15	*Aedes aegypti*	gi|157118058	1.39	< 0.001	2.28	< 0.001	Tricarboxylic acid cycle
37	myosin regulatory light chain 2 (mlc-2)	336	5	*Aedes aegypti*	gi|157167683	-1.36	< 0.001	-1.27	< 0.05	Muscle contraction
38	myosin regulatory light chain 2 (mlc-2)	259	5	*Aedes aegypti*	gi|157167683	-1.29	< 0.01	-1.23	< 0.01	Muscle contraction
39	myosin regulatory light chain 2 (mlc-2)	350	5	*Aedes aegypti*	gi|157167683	-1.47	< 0.001	-1.4	< 0.01	Muscle contraction
55	AGAP011284-PA	304	11	*Anopheles gambiae* str. PEST	gi|118779554	-1.19	< 0.05	-1.88	< 0.05	Positive regulation of translational elongation and termination
64	AGAP010657-PA	165	8	*Anopheles gambiae* str. PEST	gi|158289706	1.51	< 0.01	1.43	< 0.001	Oxygen transporter
33	myosin heavy chain, nonmuscle or smooth muscle	124	17	*Aedes aegypti*	gi|157111095	-1.54	< 0.01	-2.09	<0.01	Muscle contraction
73	myosin heavy chain, nonmuscle or smooth muscle	488	36	*Aedes aegypti*	gi|157110721	-1.4	< 0.001	1.03	< 0.01	Muscle contraction
75	glycogen phosphorylase	635	21	*Aedes aegypti*	gi|157108521	-1.41	< 0.001	-1.11	0.061	Glycogenolysis process
76	prophenoloxidase	955	38	*Anopheles gambiae*	gi|3892092	-1.46	< 0.001	-1.3	< 0.001	Oxidation-reduction process
80	AGAP007393-PB	570	19	*Anopheles gambiae* str. PEST	gi|118778070	1.9	< 0.01	1.4	< 0.05	Cell redox homeostasis / Glycerol ether metabolic process
84	citrate synthase	58	12	*Aedes aegypti*	gi|157133341	-4.15	< 0.001	-5.16	< 0.0015	Tricarboxylic acid cycle
85	isocitrate dehydrogenase cytoplasmic	263	14	*Culex quinquefasciatus*	gi|170028051	-1.51	< 0.01	-1.40	< 0.05	Tricarboxylic acid cycle
92	AGAP009218-PA	141	4	*Anopheles gambiae* str. PEST	gi|118791368	-3.87	< 0.001	-4.94	< 0.001	Proteolysis
93	AGAP005423-PA	568	11	*Anopheles gambiae* str. PEST	gi|118786445	2.33	< 0.001	2.32	< 0.001	Ubiquitin-dependent protein catabolic process
96	arginine kinase	503	14	*Mimomyia luzonensis*	gi|284159531	1.53	< 0.01	1.72	< 0.01	Transferase / Kinase
99	glutathione transferase epsilon3	358	4	*Anopheles dirus*	gi|74276396	-2.18	< 0.001	-2.83	< 0.01	Transferase
101	*cuticular protein 123, RR-2 family (AGAP003385-PA)	814	9	*Anopheles gambiae* str. PEST	gi|158290062	-1.42	< 0.001	-1.27	< 0.001	Structural constituent of the rigid cuticle

[a] Protein scores derived from Mascot algorithm, indicating identity or extensive homology ($P < 0.05$)
[b] Protein accession numbers from the National Center for Biotechnology Information non redundant (NCBInr) database
[c] Positive values mean that protein is more express in *An. gambiae* M whereas negative values mean that protein is more express in S
[d] Statistics were performed using t test, and significance was defined as $P < 0.05$
[e] Protein functions are checked using http://www.uniprot.org

Spots 09 and 21 correspond to the same protein.
Spots 33 and 73 correspond to the same protein.
Spots 37 to 39 correspond to the same protein.
* So far, the large majority of cuticular protein sequences that have been studied in arthropods have a conserved region calls the Rebers and Riddiford Consensus (R&R Consensus).

4.1. Increased sugar amounts may be indicative of water stress in *An. gambiae* females

The survival data of *An. gambiae* M and S females reared under the "rs" and "ods" conditions and further exposed to desiccating conditions suggests the existence of distinct seasonal physiological and biochemical adjustments at the emergence of the adults. The efficient management of body water content represents one of the most prominent challenges for mosquitoes during the dry season. Our results show that the dynamics of body water loss differed between females reared under "rs" and "ods" conditions in *An. gambiae* M, but no such a significant difference was observed in S form. This suggests that the S form lacks distinct phenotypes from one season to another which would enhance their desiccation resistance. Even if the accumulation of organic osmolytes like amino acids may contribute preventing body water loss (Zachariassen, 1996), we found no evidence supporting such accumulation in the 24-h-old females of either of the molecular forms reared to the "ods" conditions. Compatible solutes other than amino acids, including sugars (trehalose and sucrose),and polyols (glycerol, myo-inositol, and sorbitol) can also prevent body water loss (Elnitsky *et al.*, 2008; Thorat *et al.*, 2012; Yancey, 2005). Although, trehalose was found in small amounts regardless of molecular form or experimental condition, the concentration of circulating glucose and galactose increased in females exposed to the "ods" conditions. Increased amounts of glucose were already observed during cryoprotective dehydration in the Antarctic midge *Belgica Antarctica* (Diptera, Chironomidae) (Elnitsky *et al.*, 2008). It is, however, possible that the increased energetic demands in the 24-h-old food deprived females partly explained this increase in circulating glucose, although this would be observed in all experimental conditions. Thus, the different energy and water contents offered by carbohydrates and stored lipids may guide the determination of the most appropriate substrates that must be catabolised during periods of food deprivation and desiccation. Indeed, lipids produce more energy compared to carbohydrates (Withers, 1992), but glycogen degradation releases higher amounts of metabolic water, as it binds up to 5 times its mass in water molecules (Gibbs *et al.*, 1997; Schmidt-Nielsen, 1990). We thus suggest that the increased glucose and galactose amounts we observed in females exposed to

desiccating conditions represent the endured water stress, which is consistent with earlier data collected from *Drosophila melanogaster* (Marron *et al.*, 2003). Finally, it is also possible that mosquitoes use these two six-carbon sugars for the biosynthesis of chitin precursors, and glycan residues during cuticle differentiation (Im *et al.*, 2010; Muir and Lee, 1969).

4.2. Evidence of post-emergence adjustments of the cuticle composition in response to seasonal changes in *An. gambiae* M

In addition to the accumulation of organic and inorganic solutes in insects' hemolymph, adjustments of the cuticle composition may help mosquitoes to limit body water loss. Cuticle differentiation, starting during late embryogenesis, continues, and is soon completed after the establishment of the imaginal cuticle (Truman & Riddiford, 2000). Chemically, insects' cuticle contains proteins, peptides, and amino acids. Among these compounds, phenylalanine, tryptophan, tyrosine, and valine are particularly important for cuticle sclerotisation (Neville, 1975; Stankiewicz *et al.*, 1996; Williams *et al.*, 1987), and can be selectively favoured in the insect diet to maximise growth and cuticle synthesis (Behmer & Joern, 1993). Three (phenylalanine, tyrosine, valine) out of these four amino acids were characterised by decreased levels from 1-h-old to 24-h-old females of both M and S forms suggesting that they were at least partially used for the well-known process of cuticle settlement (Behmer & Joern, 1993; Neville, 1975; Stankiewicz *et al.*, 1996; Truman & Riddiford, 2000; Williams *et al.*, 1987).

Of particular interest, the 1-h-old females of *An. gambiae* M reared under the "ods" conditions exhibited 2- to 6-fold higher quantities of phenylalanine, tyrosine, and valine compared to counterparts reared under the "rs" conditions. Interestingly, supporting our results on body water contents, no such a difference in these aromatic amino acids was reported in *An. gambiae* S. Phenylalanine, tyrosine, and valine are known to improve the hydrophobicity of the insect cuticle (Goltsev *et al.*, 2009). Moreover, tyrosine and its derivatives assist exoskeleton hardiness by interacting with proteins (Behmer & Joern, 1993; Brunet, 1963), and also represent primary components to form sclerotin, and melanin in the newly formed insect cuticle (True, 2003). Similarly, high histidine contents were also found in 1-h-old *An. gambiae* M

females reared under the "ods" conditions, and residues of this amino acid are present in high amounts in hard (rigid) cuticle proteins (Iconomidou *et al.*, 2005).

The cuticle wax layer is cited as one of the most crucial arthropod adaptations to prevent water loss and there is evidence in the literature supporting a positive relationship among cuticle thickness, rigidity, and body water loss (Benoit *et al.*, 2010a). Higher amounts of RR-2 cuticular proteins in insects exposed to dry conditions might increase desiccation resistance by augmenting the hardness of the cuticle (Brunet, 1963; Li & Denlinger, 2009), whereas RR-1 proteins are involved in flexible ones (Willis *et al.*, 2005). In the present study, cuticular proteins 70 and 117, which belong to the RR-2 family, were 1.28- to 2.84-fold more abundant in mosquito females reared under "ods" conditions, and this may assist the level of desiccation resistance of the mosquitoes during the dry season. Arylphorins were also 1.81-fold more abundant in newly emerged *An. gambiae* M females reared under "ods" conditions. These proteins are assumed to play a significant role in the cuticle sclerotisation process, but it remains unknown if they directly act as components of cuticle structure, or if they are degraded in the cuticle for the further use of their amino acid components (König *et al.*, 1986; Telfer & Kunkel, 1991). Of note, arylphorins contain a large amount of aromatic amino acids, which supports our earlier hypothesis on the use, and thus decreased amounts of these amino acids from 1-h to 24-h-old anopheline females.

Finally, changes in cytoskeleton elements (tropomyosin, myosin light and regulatory chains) were also observed in newly emerged females of both M and S forms. Myosin light chain 1 (MLC-1), and myosin regulatory light chain 2 (MLC-2) were up-regulated in the M and S females reared under the "ods" conditions. While myosin heavy chains are well known to do the actual work in muscle contraction (Goldspink *et al.*, 1992), the role of light alkali chains remains unclear. Furthermore, our protocol did not permit us to assess whether tropomyosin belonged to the muscle or non-muscle isoform (Bullard & Pastore, 2011; Holmes, 2011). Overall, these cytoskeleton elements appear to be altered in insects exposed to a range of experimental stress conditions (Colinet *et al.*, 2012, 2013), but the functional roles of this modulation most often remain hypothetical. Further investigations will have to be

conducted to examine the exact nature of cuticle remodelling at the onset of the dry season in these mosquitoes.

4.3. Proteomics suggests a higher activity of aerobic metabolism in *An. gambiae* S

Signs of energetic metabolism adjustments were found in both molecular forms in the "ods" conditions. However, high variations in the amount of enzymes of the intermediary metabolism were observed in *An. gambiae* S. In this mosquito, the 1-h-old females were always characterised by higher amounts of enzymes involved in glycogenolytic and proteolytic processes, regardless of the experimental conditions. In particular, we found 1.41-fold more glycogen phosphorylase in the S form than in the M form under "ods" conditions. Moreover, isocitrate dehydrogenase and citrate synthase, which represent two key enzymes that strongly control the TCA cycle activity (Kültz, 2005), increased 1.6- to 4.0-fold, respectively, in the S form compared to the M form when reared in "ods" conditions. Interestingly, the two enzymes are over-expressed in colonies of the 2La karyotype of *An. gambiae* M (Cassone *et al.*, 2011) which are characterised by increased desiccation tolerance (Gray *et al.*, 2009; Rocca *et al.*, 2009).

Aspartate ammonia lyase, which participates in the fumarate metabolic process, was also 1.46-fold more abundant in the 1-h-old female *An. gambiae* S reared under the "ods" conditions, but was slightly less abundant in the M molecular form. These higher amounts of enzymes involved in energetic pathways could suggest increased energetic needs for female mosquitoes, more particularly at the onset of the dry season. Several studies have demonstrated the prominent importance of carbohydrate oxidisation, and elevated TCA cycle activity in dipterans to fuel wing muscles during flight (Briegel, 2003). Proline has been demonstrated as an essential metabolite used to fuel dipteran's flight, as in *Aedes aegypti* (Diptera= Culicidae) (Scaraffia & Wells, 2003). According to these authors, proline, which we found in higher amounts in 24-h-old *An. gambiae* S reared under "ods" conditions, is transported from the fat body to flight muscles to be converted into α-ketoglutarate. It then fuels the TCA cycle, and produces the energetic substrate necessary for striated muscle contractions. Recently, Adamou *et al.* (2011) and Lehmann *et al.* (2014) suggest that specimens of *An.*

gambiae S might migrate at the onset of the dry season towards more suitable areas. Our data could support this idea, although further studies are required to examine (i) the metabolic rate of these mosquitoes when exposed to contrasting environmental parameters (Huestis *et al.*, 2011, 2012), and (ii) if differences in the expression of the TCA cycle enzymes sustain different flying activity patterns between the two molecular forms, particularly at the onset of the dry season.

5. CONCLUSION

Ecological divergence and contrasted population dynamics in the M and S forms of *An. gambiae* in Sahelian areas of sub-Saharan Africa suggest that the two species adopted different dry-season survival strategies to overcome the detrimental effects of climatic seasonality (Adamou *et al.*, 2011; Lehmann *et al.*, 2010, 2014). Supporting our expectations, the present study provides evidence that developmental acclimation contributed to alternating phenotypes between rainy and dry season in freshly emerged mosquitoes, and these phenotypes differed between the two molecular forms. In particular we found evidence that these alternating phenotypes enhance tolerance and resistance to desiccation in both forms and are accompanied by several changes in physiological and biochemical processes, including an increase in the biochemical hydrophobicity of the cuticle in the M form and increased demand of energetic substrate in the S form. Further research on the metabolic pathways and their underlying regulatory mechanisms that are involved in preparing individual mosquitoes for the adverse dry season conditions are needed to better understand population dynamic observations conducted at the population level. Combining experimental studies with high-throughput methods such as proteomics and metabolomics holds much promise towards elucidating the physiological mechanisms underlying dry season tolerance in these tropical mosquitoes. Moreover, such studies will pave the way for the development and successful implementation of innovative vector control strategies that target mosquito populations during unsuitable periods when they are most amenable to control.

Acknowledgements

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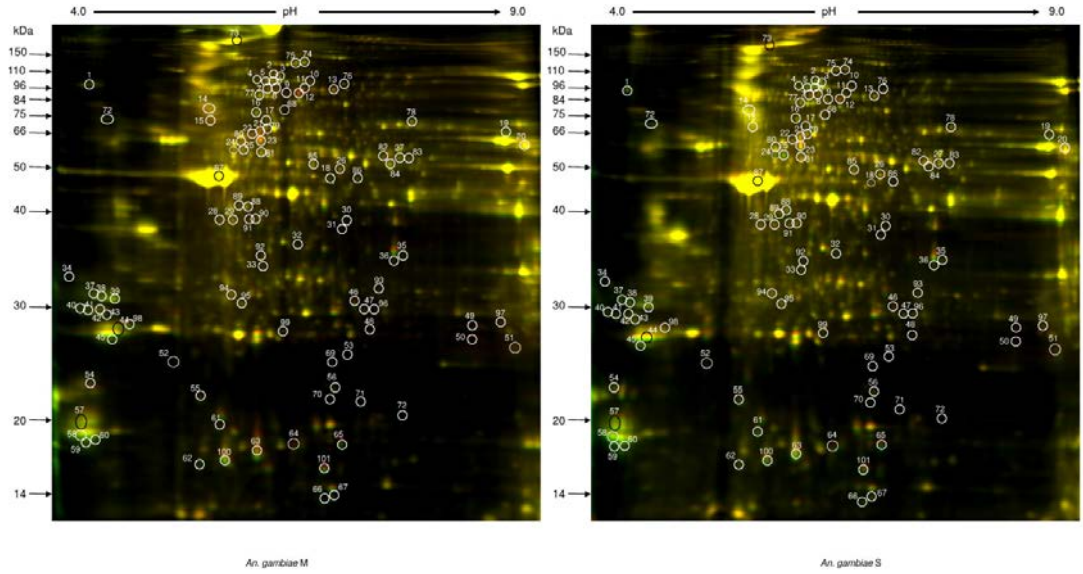
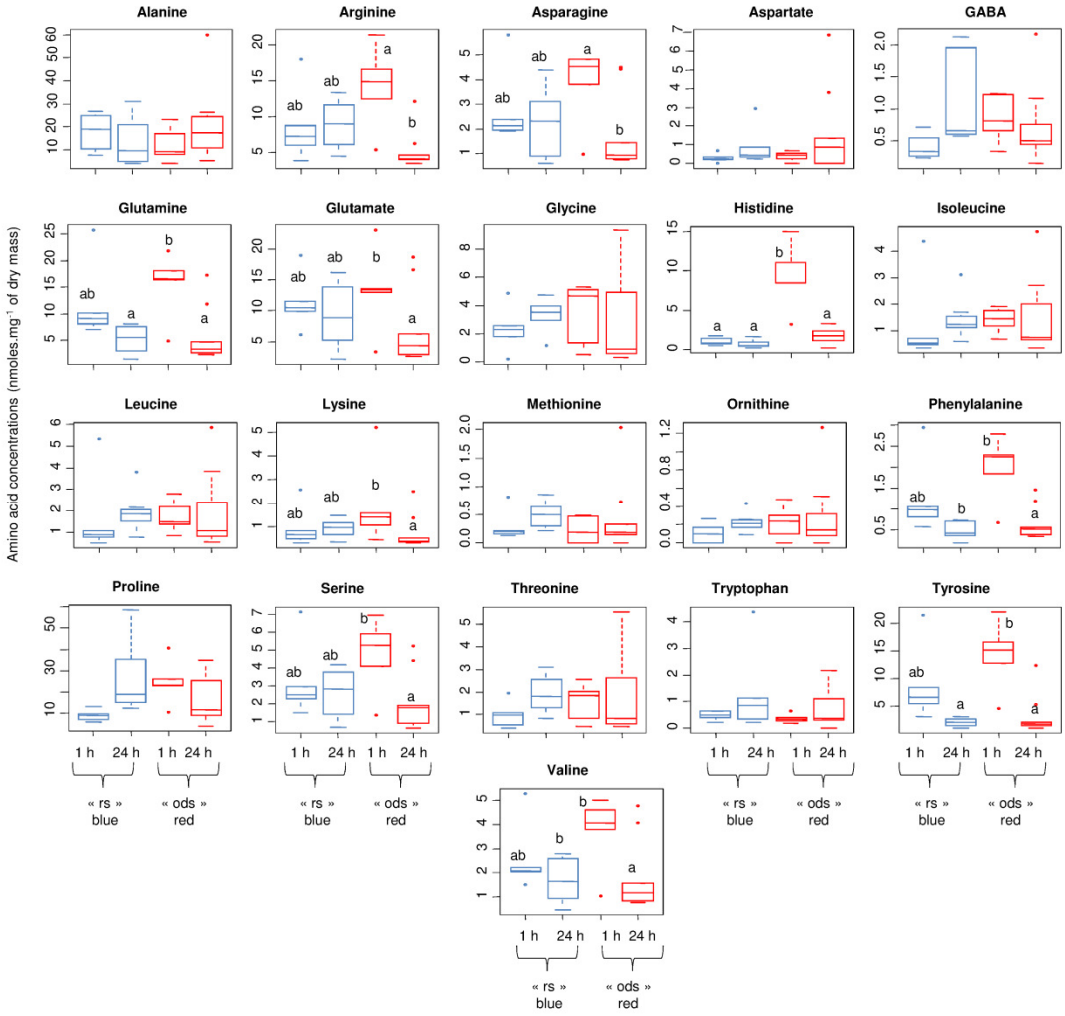
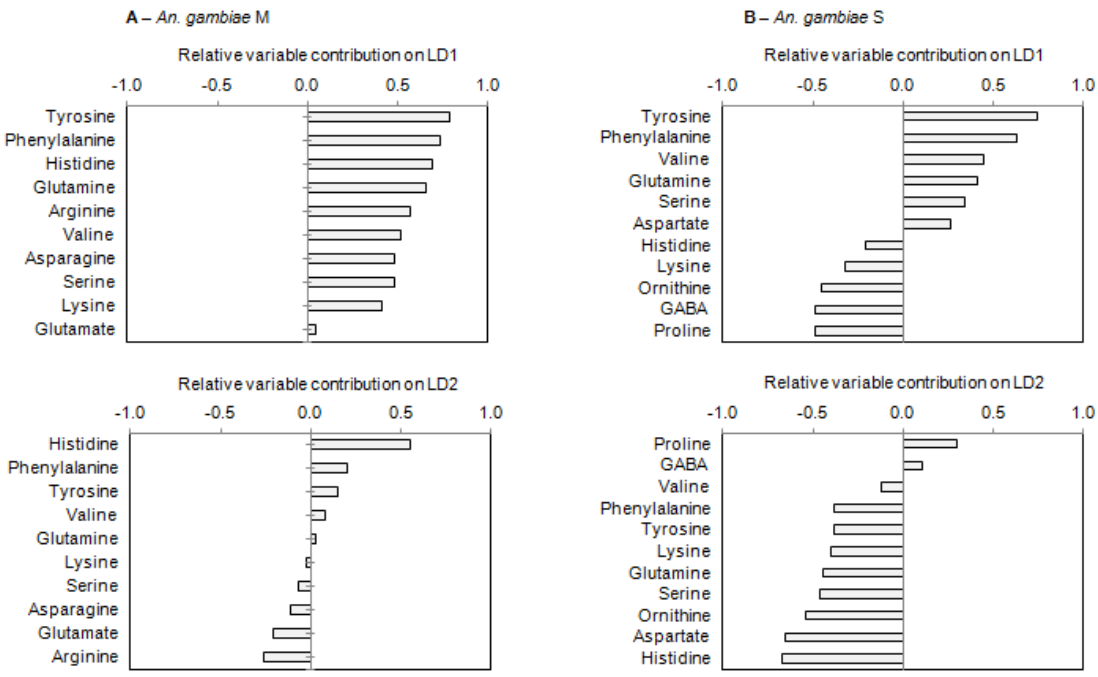
SUPPLEMENTARY DATA



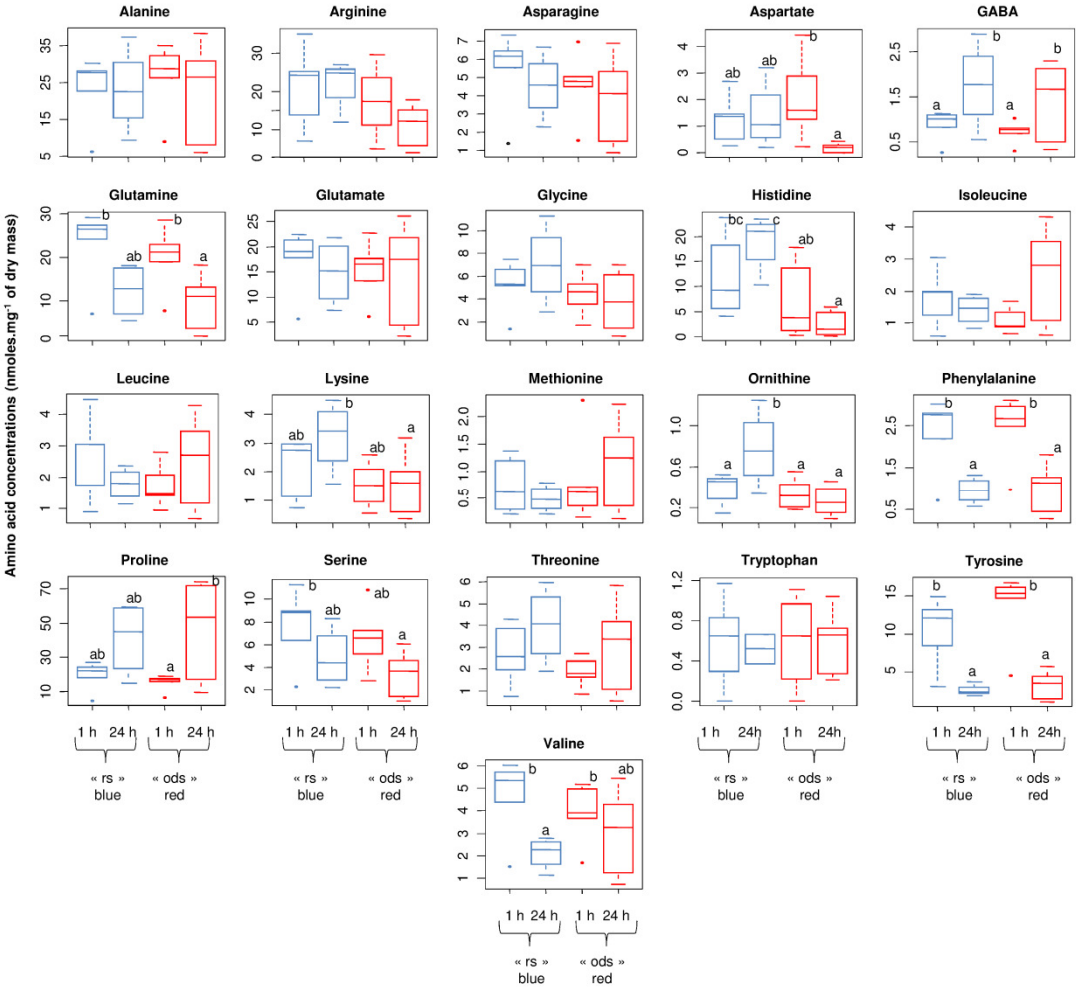
Supplementary data 1 Representative image of the two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) in *An. gambiae* S (right) and *An. gambiae* M (left). Females reared under the "ods" conditions were labeled with Cy3 (green), whereas females reared under the "rs" conditions were labeled with Cy5 (red).



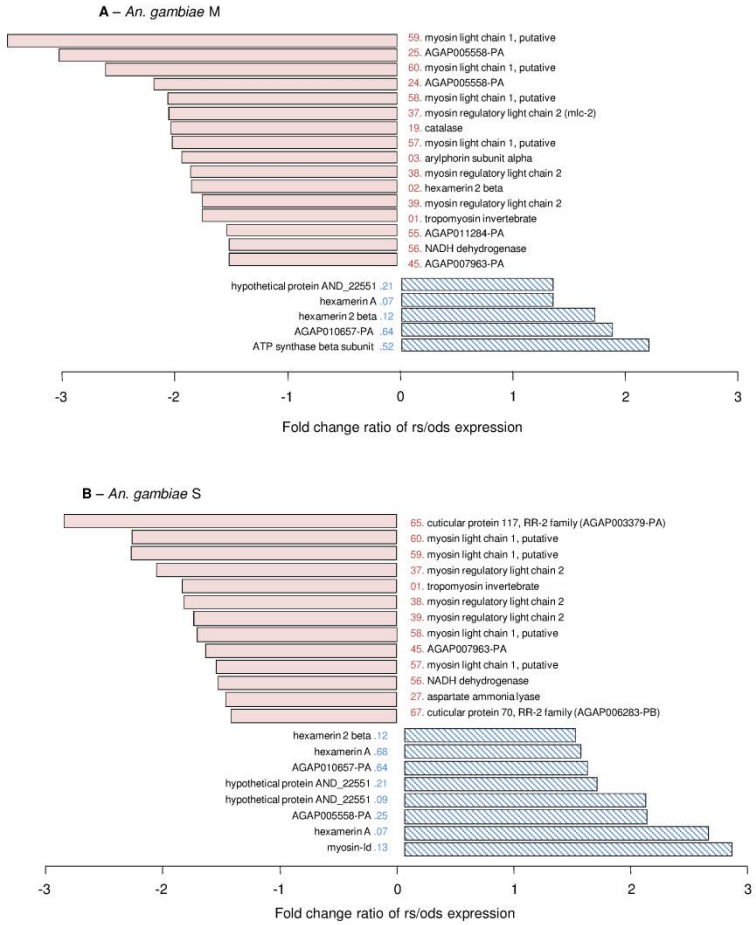
Supplementary data 2 Boxplot of the amino acid concentrations (nmoles mg^{-1} of dry mass ± s.e) in 1-h-old and 24-h-old *An. gambiae* M reared under "rs" (blue) or "ods" (red) conditions. Different letters above a box indicate significant differences among the experimental conditions (two-way ANOVAs, experimental conditions and females' age) based on adjustment with the Benjamini-Hochberg algorithm followed by Tukey *post-hoc* comparisons. No letter indicates non-significant differences in amino acid amounts among the experimental groups.



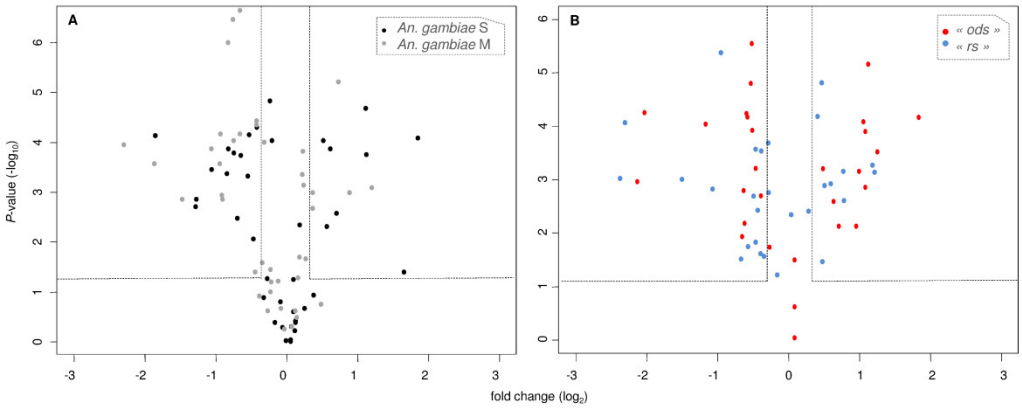
Supplementary data 3 Relative contribution of each amino acid to LD1 and LD2 in An. gambiae M and S.



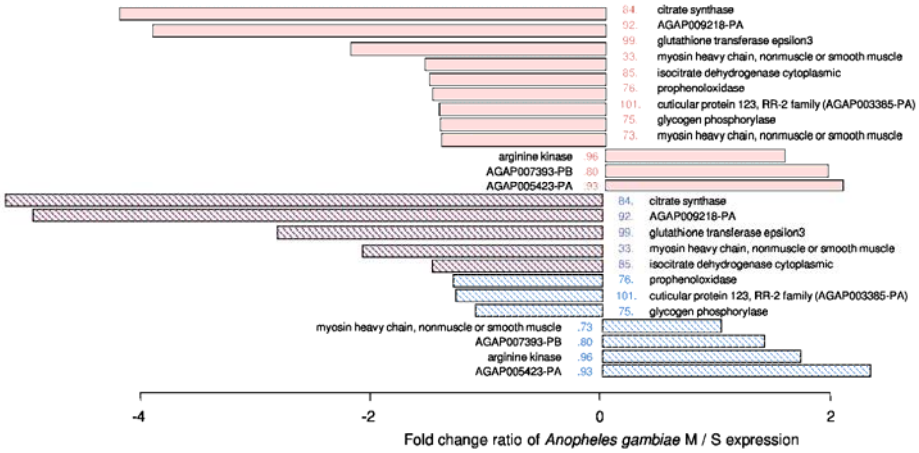
Supplementary data 4 Boxplot of the amino acid concentrations (nmoles mg^{-1} of dry mass ± s.e) in 1-h-old and 24-h-old Anopheles gambiae S reared under "rs" (blue) and "ods" (red) conditions. Different letters above a box indicate significant differences among the experimental conditions (two-way ANOVAs, seasonal conditions and females' age) based on adjustment with the Benjamini-Hochberg algorithm followed by Tukey post-hoc comparisons. The absence of a letter indicates non-significant amino acid amount differences among the experimental groups.



Supplementary data 5 Differential fold-change of the 21 selected spot proteins between “rs” and “ods” conditions in 1-h-old females of Anopheles gambiae M and S. Blue dashed bars are positive values that represent spots that were more abundant in “rs” conditions, whereas red bars are negative values that represent spots that were more abundant in “ods” conditions.



Supplementary data 6 Volcano plot of spot protein significance against their relative fold-change (A) between the experimental "ods" and "rs" conditions in 1-h-old females of *Anopheles gambiae* S (black) and M (grey) and (B) between*Anopheles gambiae* S and M reared under "ods" (red) and "rs" (blue) conditions. Each point represents one spot. The X-axis displays the $\log_2$-transformed fold-change= positive fold-changes represent higher protein expression in the "rs" conditions (A) or in the S form (B), whereas negative fold-changes represent higher protein expression in the "ods" conditions (A) or in the M form (B). The Y-axis displays $\log_{10}$-transformed *P*-values of differential protein expression associated with Student's*t*-tests. The horizontal and vertical black dashed lines indicate the threshold for significance ($P < 0.05$) and an average-fold difference of at least 1.3 (absolute value), respectively.



Supplementary data 7 Differential fold-change of the 24 selected spot proteins between M and S forms reared under "rs" (dashed blue bars) and "ods" (solid red bars) conditions. Positive values represent spots that were more abundant in*Anopheles gambiae* M, whereas negative values represent spots that were more abundant in *Anopheles gambiae* S.

2. Ajustements du métabolisme énergétique chez les femelles *An. coluzzii* et *An. gambiae* (Article II)

Nos précédents travaux ont montré que des mécanismes de réponses sont établis par les femelles anophèles au début de la saison sèche et que ces mécanismes diffèrent entre *An. coluzzii* et *An. gambiae* (Article I). Tandis que les femelles *An. coluzzii* montrent des ajustements biochimiques visiblement liés à la perméabilité de la cuticule, les femelles *An. gambiae* montrent une sur-expression de métabolites et d'enzymes impliqués dans le métabolisme énergétique. Ces résultats suggèrent notamment que les femelles *An. gambiae* ont des besoins en substrats énergétiques plus importants. Des ajustements au niveau du métabolisme énergétique semblent donc être impliqués dans la réponse des femelles Anophèles aux conditions contraignantes de la saison sèche. Nous avons donc voulu examiner les changements du métabolisme énergétique de ces espèces associés aux changements des conditions environnementales perçues par les femelles *An. coluzzii* et *An. gambiae*. Par ailleurs, en conditions contraignantes, une dépression de l'activité métabolique de ces femelles, peut constituer un marqueur de diapause hivernale chez les insectes (Hahn & Denlinger 2007, 2011 pour des revues). Cette dépression de l'activité métabolique devrait donc être observée au début de la saison sèche chez les femelles *An. coluzzii* si elles estivent pendant la saison sèche (Huestis & Lehmann, 2014). Pour *An. gambiae*, nous attendons plutôt une augmentation de l'activité métabolique d'après les résultats précédemment obtenus (Article I), mais aussi d'après la stratégie de survie que cette espèce est soupçonnée développer (*i.e.* dispersion/migration vers des habitats ou microhabitats favorables). Les variations de l'activité métabolique des femelles Anophèles ont été mesurées indirectement à travers l'évaluation de la quantité de molécules de dioxyde de carbones émises par les femelles Anophèles à l'aide d'un respiromètre. Cet outil nous permet également de mesurer la quantité d'eau transpirée par les insectes de telle sorte que nous avons pu mettre en relation les variations de l'activité métabolique avec la dessiccation des insectes. Les analyses ont été effectuées sur des femelles *An. coluzzii* et *An. gambiae* exposées à des conditions reproduisant la saison des pluies (RS) ou le début de la saison sèche (ODS). La régulation du métabolisme énergétique de ces deux espèces a été également suivie en RS et ODS,

par dosage de l'expression des gènes des hormones adipokinétiques (AKH). Ces gènes mobilisent et transportent le substrat énergétique des organismes du corps gras à l'hémolymphe et sont connus, par ailleurs, pour leur implication dans la mobilisation du substrat énergétique dans les muscles alaires (Aresse & Soulages, 2010). L'expression de ces gènes permettrait de mettre en évidence une meilleure capacité à disperser chez les femelles, notamment *An. gambiae*, au début de la saison sèche. De récentes suspicions établissent que les AKH pourraient également être impliqués dans la régulation du métabolisme énergétique pendant la diapause des insectes (Hahn & Denlinger, 2011 ; Isaebelle *et al.*, 2005). L'expression des ces hormones pourraient alors également renseigner une stratégie de dormance chez les femelles, notamment *An. coluzzii*. Des analyses de l'allométrie des insectes et de la fréquence de vol de femelles ont également été analysées en relation avec leur activité métabolique et leur transpiration. En effet, ces variables sont connues pour influencer l'activité métabolique des insectes.

Ces études ont été réalisées sur deux populations d'*An. coluzzii* exploitant des gîtes larvaires présents ou non pendant la saison sèche. La variation des habitats de ces espèces pourraient potentiellement affectés de façon différentielle la régulation du métabolisme de ces organismes et, suggérant ainsi une variabilité de la plasticité phénotypique de ces femelles.

Les résultats obtenus feront prochainement l'objet d'une publication dans « Insect Biochemistry and Molecular Biology ».

Metabolic rate and AKH peptides variations in females Anopheles coluzzii and An. gambiae during the dry season - (Article II)

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Abstract

Females of *Anopheles coluzzii* and *An. gambiae* are the major malaria vectors of West Africa. In this area, mosquitoes are exposed to high temperatures and low values of relative humidity during the dry season. Aestivation and migration from/to distant suitable locations have been proposed as mechanisms that allow *An. coluzzii* and *An. gambiae*, respectively, to persist throughout the dry season and rapidly re-colonize the area at the onset of the rains. However, studies to date failed to identify dormant or migrant phenotypes. In this study we used two populations of *An. coluzzii* displaying permanent and temporary dynamics in the fields and one temporary population of *An. gambiae* to explore several phenotypic traits known from other insect species to be involved in aestivation or migration strategies. Hence, changes in metabolic rate (CO_2 emission), surface to volume ratio, flight activity and water losses were measured in mosquitoes experimentally exposed to the contrasted conditions of the rainy season (RS) and the onset of the dry season (ODS) they experience in the fields. Moreover, we measured expression profiles of genes coding for AKH peptides as these are known to be involved in metabolites' storage and mobilisation. Our results showed distinct phenotypes among the species/populations in response to dry season conditions: (i) CO_2 emissions were increased in *An, coluzzii* but not *An. gambiae*, (ii) H_2O releases did not follow the metabolic rates but drastically decreased in all populations, (iii) in *An. gambiae,* decrease of water loss could be due, at least in part, to a diminution of the surface to volume ratio, (iv) expression patterns of AKH genes were similar among species from temporary populations but different from those of permanent one. Hence, contrasted phenotypic profiles emerged in the different species and populations in response to the dry season. Significance of our results was discussed in the light of recent studies in the field.

Keywords: *Anopheles gambiae s.l;,* dry season, metabolic rate; water loss; AKH; surface to volume ratio

Abbreviations: RS = Rainy season; ODS = Onset of the dry season; VCO2= Rate of carbon dioxide release; VH2O = Rate of water loss; WL = Wing length.

1. INTRODUCTION

In West Africa, *An. coluzzii* (previously known as "M molecular form", Coetzee *et al.*, 2013; Lee *et al.*, 2013) and *An. gambiae* (previously known as "S molecular form") are the main malaria vectors. In dry savannahs, both species exploit temporary water ponds (*i.e.* larval breeding-sites) maintained only during the rainy season, as they dried-up at the onset of the dry season (Gimonneau *et al.*, 2012). In such areas, the mosquito densities are highly seasonal, decreasing drastically at the onset of the dry season and increasing exponentially as soon as the rain returns (Baldet *et al.*, 2003; Costantini *et al.*, 2009; Diabate *et al.*, 2004, 2002). Interestingly however, the population densities of *An. coluzzii* only can be maintained all year long as they can also exploit large anthropogenic (*e.g.* dams, ricefields, etc.) or natural (*e.g.* ponds, river edges, etc.) water ponds preserved throughout the year (Costantini *et al.*, 2009; Gimonneau *et al.*, 2012). Depending on the nature of their larval breeding sites, mosquito *An. coluzzii* and *An. gambiae* may have thus developed distinct mechanisms to survive the desiccating conditions of the dry season. To date, the mechanisms by which mosquitoes survive the dry season is still controversial, but two strategies have been proposed based on genetics and population dynamics studies (Adamou *et al.*, 2011; Huestis & Lehmann, 2014; Lehmann *et al.*, 2010, 2014; Simard *et al.*, 2001). It is proposed that females *An. coluzzii* survive the desiccating conditions of the dry season in a dormant state known as the aestivation (Huestis & Lehmann, 2014; Lehmann *et al.*, 2010; Yaro *et al.*, 2012) whereas *An. gambiae* would display high dispersion abilities to rapidly colonize favourable habitats at the onset of the dry season or habitats becoming suitable when the rains start (Adamou et al, 2011; Huestis and Lehmann, 2014). For *An. coluzzii* however, mitigated phenotypes are observed depending on the characteristics of the breeding sites: weak aestivators or strong aestivators can be found in areas where breeding sites are temporary or permanent, respectively (Yaro *et al.*, 2012).

If species express aestivating and/or dispersing strategies, specific adjustments of their physiological traits should be observed depending on environmental conditions. In particular, generalisation from previous works on overwintering diapause points toward a metabolic rate depression as one of the most significant

physiological processes characterising aestivating insects (Hahn & Denlinger, 2011, 2007; Storey & Storey, 1990). For *An. gambiae*, which is thought to show higher dispersive abilities, we await for an increase in metabolic rate to provide energetic substrates needed for sustained flight (Van der Horst, 2003). Previous work failed to link metabolic rate depression with aestivation in females *An. coluzzii* in the fields during the dry season (Huestis *et al.*, 2012, 2011). However, because metabolic rate is strongly influenced by the individual's age and its trophic and reproductive status (Nespolo *et al.*, 2003; Rogowitz & Chappell, 2000), we assume that metabolic rate modifications might be more reliably evidenced using mosquito reared under controlled and normalised environmental and biological conditions from larval to adult development. In arid environments, a decrease in metabolic rate has already been observed to help reducing water losses and increase the insect's fitness (Chown, 2002; Hadley, 1994; Rourke, 2000). For instance, the metabolic rate of the Mediterranean tiger moth *Cymbalophora pudica* (Lepidoptera, Arctiidae) rapidly decreases when individuals are exposed to desiccating conditions, helping them to reduce body water loses and increase longevity under these conditions (Koštál *et al.*, 1998).

Other mechanisms can be involved to prevent body water loss in insects. Among these, changes in body size and mass may affect the surface to volume ratio of specimens. According to biophysical theories, body water loss by an individual is reduced when its surface area to volume ratio is reduced (Hadley, 1994). Hence, the decrease of this ratio is described as one of the most common mechanism for reducing water loss in insects (Benoit & Denlinger, 2007; Hadley, 1994). For instance, the epedaphic collembolan species reduce its surface area to volume ratio in order to challenge the stressful dehydrating conditions of its environment (Kaersgaard *et al.*, 2004).

Preventing body water loss also involves accumulation of specific organic compounds or catabolism of body reserves, like stored glycogen. Catabolism of glycogen generates significantly higher amount of bounded water than any other body substrate (Schmidt-Nielsen, 1990) as it releases up to five-times its mass in water molecules (Gibbs, 2002; Gibbs *et al.*, 1997; Schmidt-Nielsen, 1990). It is interesting to mention that glycogen degradation also provides precursors for the synthesis of osmoprotectants in the form of sugars, polyols or amino acids (Danks, 2000; Hahn &

Denlinger, 2007). Degradation of glycogen is ensured by a glycogen phosphorylase of which the regulation is under the control of the adipokinetic hormones (AKH; Gäde, 2004; Wilps & Gäde, 1990; Ziegler *et al.*, 2011). The expression of AKH peptides in insects is also well documented in the context of flight maintenance because these peptides supply the energetic substrates needed for striated muscle contraction in insects (Arrese & Soulages, 2010; Van der Horst, 2003). An increased expression of these peptides in female *An. gambiae* might thus help us to identify a "dispersive" phenotype. Moreover, recent investigation suggests that these peptides also allow the regulation of energetic metabolism in overwintering diapause insects (Hahn & Denlinger, 2011). Therefore, increase expression of these peptides might also be considered as an aestivation marker at the onset of the dry season.

Here, we evaluated the seasonal variations in allometry (*i.e.* body size and mass), metabolic rate, water loss and AKH peptides (Anoga-AKH-I, Anoga-AKH-II and their putative receptor Anoga-AKH-R) gene expressions in females *An. coluzzii* from both permanent and temporary breeding-sites and in females *An. gambiae*. Insects were experimentally subjected to the environmental conditions they experience during the rainy (RS) and the onset of the dry season (ODS) in the Burkina-Faso. All the phenotypic traits were measured and compared between the two experimental rearing conditions and among species/populations. Metabolic rate was indirectly measured using the carbon dioxide content release by females submitted to the hottest and driest hours of the day of both RS and ODS conditions during which females are supposed to be resting.

Extrapolating from what is known about the species/populations dynamics in the fields, we made the following predictions: (i) the females of *An. coluzzii* but not *An. gambiae* will decrease their metabolic rates, (ii) a decrease of the water losses will be observed at least in *An. coluzzii* (iii) a decrease of the surface to volume ratio can be expected in all species/populations to help reduce water losses (iii) differential AKH gene expressions between species will highlight different metabolic mobilisation and, hence, different survival strategies, and (iv) distinct phenotypes between temporary and permanent populations of *An. coluzzii* will be observed as markers to distinct aestivation abilities, as supposed by Yaro *et al.* (2012).

2. MATERIALS AND METHODS

2.1. Mosquito populations and rearing conditions

2.1.1. Mosquito populations

Experiments were conducted using two mosquito populations of *An. coluzzii* from the Burkina-Faso founded in September-October 2012 using up to 50 gravid females collected in two sites where mosquito populations displayed different dynamics. The first site was the village of Bama [11°23'N, 04°24'W], where *An. coluzzii* is the main encountered species (Gimonneau *et al.*, 2012) and is permanent, breeding all year long in rice fields. The second site was the village of Soumousso [11°01N, 04°02'W], where a seasonal pattern of *An. coluzzii* population dynamics has been reported (Dabiré *et al.*, 2007). A third population used in this study was established at the IRSS insectary in 2009 from *An. gambiae* females of temporary populations collected in the village of Soumousso as well. All populations were maintained under the same insectary conditions (27ºC, 70% humidity, light/dark cycles of 12/12h) prior to the analysis, which were conducted in 2013. Populations were maintained in separate rearing rooms to avoid any crossbreeding.

2.1.2. Environmental conditions

The three mosquito populations were reared from eggs to adults in two programmable climatic chambers (Binder KBF720 VWR international S.A.S), programmed to reproduce the natural daily fluctuations of environmental conditions experienced by mosquitoes during the rainy season (hereafter referred as the RS condition) and at the onset of the dry season (hereafter referred as the ODS condition). Conditions were identical to those used in previous studies (Article I; Mamai *et al.*, 2014; **Fig. 1**), except for photoperiod variation between seasons, which was considered this time. Variations of day length were accounted for using hourly-recorded light intensity data during the RS and ODS conditions using a weather monitoring station (Weatherlink; Davis Instruments, Hayward, CA, U.S.A.). Climatic conditions in the incubators mimicked those observed in the fields and were tightly monitored using MicroLog Pro monitors placed inside the incubators (EC750, Davis Instruments, Hayward, CA, USA).

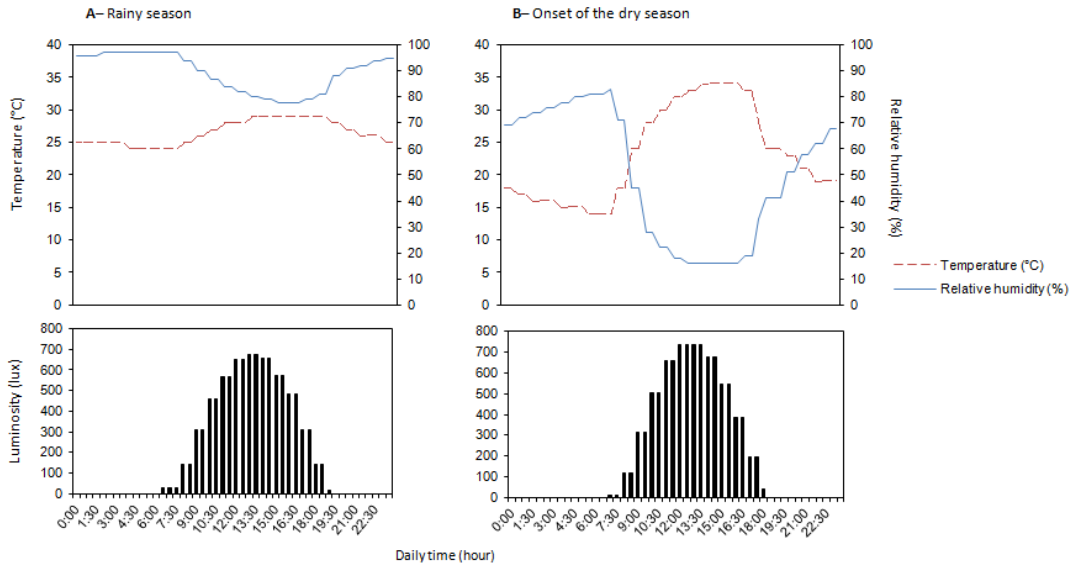

Fig. 1 Daily fluctuations of temperature (°C – dashed red line), relative humidity (% – blue line) and light intensity (black bars) programmed to simulate the environmental variations of (A) the rainy season (RS), and (B) the onset of the dry season (ODS) within the climatic chambers.

2.1.3. Mosquito rearing

Eggs from each population were synchronously collected from more than 50 caged females at the IRSS of the Burkina-Faso and sent to the IRD of Montpellier (France). Three independent batches of eggs, each time laid by different caged females, were used for this study. Climatic chambers were switched between the batches to account for any potential "chamber effect". Rearing conditions were the same as previously described (Article I; Mamai *et al.*, 2014). Upon emergence, females only were maintained within the climatic chambers, in small cages at a density of 30 females per cage, until they reached four to six-days old. Females were fed *ad libitum* with glucose (10%) and water. Twenty-four hours before the experiments, females were food and water deprived in order to avoid any potential bias in carbon dioxide (VC02) and water loss rate (VH2O) measures due to the presence of different amounts of sugars and water in the females' gut.

2.2. Metabolic rate (VCO2) and body water transpiration (VH2O)

The VCO2 and VH2O rate of females from the three populations were measured using a flow through respirometry and a CO_2-H_2O gas analyser (Sable System, Li-7000 CO_2/H_2O infrared gas analyzer, Li-Cor-Biosciences, Lincoln, NE). For each population and each experimental condition (RS, ODS), 6 to 9 runs were performed on

randomized pools of 4 females (so that the minimum identification threshold within the gas analyzer system was reached). Four to six-days old females were placed into a 200 mm x 400 mm (L x l) glass chamber. The chamber was then connected to the gas analyzer and flushed with a constant flow of air at a rate of 200 mL/min. The temperature and relative humidity of the air entering the chamber were modulated using a Peltier effect temperature controller and a Dew point generator/Relative humidity controller system, respectively. The programmed conditions corresponded to the ones monitored during a 4 hours time frame corresponding to the hottest and driest period of the day for each season during which females are resting (from noon to 4 p.m). Hence, the relative humidity and temperature of the flowing air were respectively 18% and 34 ºC for the ODS, or 70% and 29 ºC for the RS. The same time frame (*i.e.* from noon to 4 p.m) was considered for sampling the mosquitoes for both environmental conditions to account for their endogenous rhythms. Lastly, mosquitoes were recorded in the chamber using a HD Camera (Sanyo, model) to check for any activity, which could impact the rate of CO2 or H2O released. We considered only the records where continuous observation of the mosquitoes could be made through a complete measurement sequence (see below).

Each run lasted 100 min during which both the rate of CO_2 and H_2O releases (VCO2 and VH2O) were recorded. Following an acclimation period of 25 min, measurements of VCO2 and VH2O were performed for 15 min. The measurement sequence was repeated three times. Control measures were performed in an identical parallel system without mosquitoes for 10 min before each measurement sequence (*i.e.* 3 x 10 min). Data were analyzed using the Expedata software (Sable System International V.1.0.1). Amounts of mosquitoes' VCO2 and VH2O were calculated by subtracting the control values to those measured with mosquitoes in the chamber. After each run, viability of the females was checked before they were snap-frizzed, dried for 3 days in a 60 ºC oven, and weighed on a precision microbalance (Sartorius, 0.1mg accuracy) to determine the dry mass of each sample. For each run, VCO2 and VH2O were averaged per 15 min period and expressed in µl/min/mg of mosquito dry mass to assess for mass variability between species and conditions, as previously described (*i.e.* Gray & Bradley, 2006; Huestis *et al.*, 2011, 2012).

Body size was also estimated using the wing length (WL; **Supplementary**

data 1). The right wing of each female was removed from the thorax before mosquitoes were dried. Wings were then mounted on a microscope slide for further processing. Pictures of mounted wings were taken under a dissecting microscope (x 20, Leica DFC425) and the wing length was measured from the allula to the wing tip as described previously by Charlwood (1996). The measures were performed using the ImageJ1.41.0 software (Wayne Rasband, National Institute of Health, Rockville, MD, U.S.A.) to an accuracy of ±0.001 mm. Damaged wings were discarded, giving a total of 172 wings analysed. WL was divided by the dry mass of mosquitoes to estimate the surface to volume ratio.

2.3. Evaluation of the *AKH*-related genes expression

2.3.1. RNA extraction and cDNA synthesis

For each population, and each experimental condition (RS, ODS), 3 to 4 replicates of 10 randomly pooled 4 days-old female mosquitoes were analysed. Collected mosquitoes were immediately snap-frozen in liquid nitrogen, and conserved at -80 °C within RNA*later* solution (Ambion, USA) until further processing.

The RNA*later* solution was removed and the total RNAs of each sample were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) coupled with the RNeasy Kit (QIAGEN). Samples were thereafter treated with DNase I (Ambion, USA) in accordance with the manufacturer's instructions, and quantified by spectrophotometry at 260 nm (Nanodrop2000, Thermo Scientific). Single-stranded cDNAs were then synthesized from total RNAs with Superscript II reverse transcriptase (Gibco BRL, Invitrogen) as described in (Bigot *et al.*, 2012) and according to the manufacturer's instructions.

2.3.2. Real-time quantitative PCRs

All Real-time quantitative PCRs were conducted as described in Bigot *et al.* (2012). A total of ten genes (*Actine*, *Rps13*, *Rps7*, *Rpl5*, *h3a, Cytp450, Tubulin, hsp83*, *EGFR*, and *18s*) were tested as putative housekeeping genes. Following a BestKeeper analysis (Pfaffl *et al.*, 2004), the Rps13 was selected as the reference gene since its expression was stable in all samples whatever the experimental condition or anopheline population tested. Further, mRNA of the two AKHs genes *Anoga-AKH-I* and *Anoga-*

AKH-II, and the AKH receptor gene *Anoga-AKH-Receptor* (see Kaufmann and Brown, 2006) were examined. Specific primers (reverse and forward) for both housekeeping genes and target genes were designed using the Eprimer3 software (http://emboss.bioinformatics.nl/cgi-bin/emboss/eprimer3; **Table 1**).

Each PCR reaction was technically triplicated and consisted of 6 µL of absolute Blue SYBR Green Fluor (ROCHE Molecular Systems Inc., USA), 2 µL of cDNA (25 ng.µl^{-1}), 0.5 µL of each reverse and forward primers (10 µM), and 3 µL of RNA free water. The qPCR program and conditions was the same as the one described in Bigot *et al.* (2012). The cycle threshold values (Ct-values) for both reference and target genes were determined using the Light-Cycler® 480 software (Roche, FRANCE). The average Ct value of each technical triplicate was used to normalise candidate gene expression levels to the geometric mean of the reference gene level using the Q-Gene software (Simon, 2003).

Table 1. Nucleotide sequences of primers used in qPCR reactions for the amplification of Actine, Rpl13, Rpl7, Rpl5, NADPH, hsp83, h3a, and 18s , *Anoga-AKH-I, Anoga-AKH*-II and *Anoga-AKH-Receptor* in *An. gambiae*.

Primer	Direction	Séquences (5'-3')
Actine	FOR	CTGGACTTCGAGCAGGAGAT
Actine	REV	CGCACTTCATGATCGAGTTG
Rps13	FOR	TATTTCCAAATCCGCGCTAC
Rps13	REV	CATGATACGCAGCACCTTGT
Rps7	FOR	ACCCCAACAAGCAGAAGAGA
Rps7	REV	TACACCGACGCAAAAGTGTC
Rpl5	FOR	GGACTGAACATTCCGCACTC
Rpl5	REV	GATGCCCAGCGAGATGTACT
h3a	FOR	ATCCGTCGGTACCAGAAGTC
h3a	REV	AATGTCCTTCGGCATAATGG
CytP450	FOR	TACCAAATGAAGGGCATGGT
CytP450	REV	AACACCGCGTAATTCAAACC
Tubulin	FOR	AAGCTCGAATTCGCCATCTA
Tubulin	REV	CCAATCAAACGGTTCAGGTT
hsp83	FOR	CTGCGTGAGTTGATCTCGAA
hsp83	REV	ATCGTTCCGAGGTTGTTCAC
EGFR	FOR	GGGAATGTTGCCATCTGTTC
EGFR	REV	GACATTTCCGTACGCAGGTT
18s	FOR	ACCCGCGTCACTACAAAATC
18s	REV	CGGTAGTTTTCGTGTGCTGA
Anoga-AKH-I	FOR	TGCTGATTTGTGCCTCTTTG
Anoga-AKH-I	REV	ATTCCCCAACCCTACCTGAA
Anoga-AKH-II	FOR	CGCTGGACAGGTAACGTTTT
Anoga-AKH-II	REV	GACTCATCCGTTTGCAGTGA
Anoga-AKH-R	FOR	CGTACTATGCGAACGAAACG
Anoga-AKH-R	REV	TGCGCCAACATGATATTGAT

2.4. Data analysis

All statistical procedures were conducted using the *R* 3.1.1 statistical software (R Development Core Team, 2008). Before analysis, rearing session effect was test using ANOVA model. Then the normal data distribution and homoscedasticity of each dataset were monitored using Shapiro-Wilk and Bartlett's tests, respectively. Accordingly, parametric or non-parametric tests were further processed.

A two ways ANOVA analysis was first performed to test the variation of allometric measures (dry mass, wing length and surface to volume ratio) and the flight activity of females between the two environmental conditions (RS, ODS) and among the three populations. When needed, Tukey HSD procedure was used to perform *post-hoc* comparisons among the levels of significant factors.

Generalised linear models (quasipoisson error and logit function) were performed to investigate the variations of metabolic rate (VCO2; expressed as µl/min/mg of dry mass) in function of the two environmental conditions, the three anopheline populations, the flight, the wing size and the surface to volume ratio as explanatory variables. A second model was used to investigate the female's water loss (VH2O, expressed as µl/min/mg of dry mass) in function of the two environmental conditions, the three anopheline colonies, the metabolic rate (VCO2, expressed as µl/min/mg of dry mass), the flight, the wing size and the surface to volume ratio as explanatory variables. Because of the significant interaction term "surface to volume ratio : environmental conditions", linear regressions with Pearson correlation tests were performed to further examine the relationships between the both variables in females when reared under RS or ODS conditions. For both models main effects and all relevant first and second order interactions were tested in full models. Model simplification used stepwise removal of terms, where the significance of the terms was estimated using the difference in Akaike's information criterion (AIC).

The expression level variations of *Anoga-AKH-I*, *Anoga-AKH-II* and *Anoga-AKH-R* genes were analysed between the two experimental conditions and among the three populations using two way ANOVAs. Statistical significance was set at α=0.05, and *post-hoc* comparisons were performed when required.

3. RESULTS

3.1. Allometric and flight activity variations

Analyses showed that both wing size and female's dry mass significantly differed among the three Anopheles populations (ANOVA, *ddl*=2, F_{size}=12.91, F_{mass}=16.02, *P*<0.001; **Fig. 2A-B**). Of both variables, only the dry mass was significantly influenced by the environmental conditions (ANOVA, *ddl*=1, F=5.4, *P*<0.05; **Fig. 3B**), and this effect was mainly due to the significant increase of the dry mass of *An. gambiae* females reared in ODS conditions by comparison to those reared in RS ones (**Fig. 2B**). Overall, the surface to volume ratio of females differed among populations (ANOVA, *ddl*=1, F=13.0, *P*<0.001, **Fig. 2C**) but not between environmental conditions (ANOVA, *ddl*=1, F=0.26, *P*=0.61). However, a significant interaction between environmental conditions and populations was observed (ANOVA, *ddl*=2, F=4.8, *P*<0.05), suggesting that the impact of the environmental conditions on the surface to volume ratio differed among the populations. Indeed, only females of *An. gambiae* exhibited significant changes, with the ones reared under ODS conditions exhibiting a 1.8 fold lower surface to volume ratio than females reared under RS conditions (**Fig. 2C**). This decrease of the ratio is due to the significant increase of dry mass in females reared under ODS conditions (see above, **Fig. 2B**).

Overall, the flight activity was the same between the populations (ANOVA, *ddl*=2, F=0.33, *P*=0.72). An increase of the flight activity can be noticed for mosquitoes submitted to ODS conditions, but this trend was not significant (ANOVA, *ddl*=1, F=2.79, *P*=0.11, **Fig. 3**).

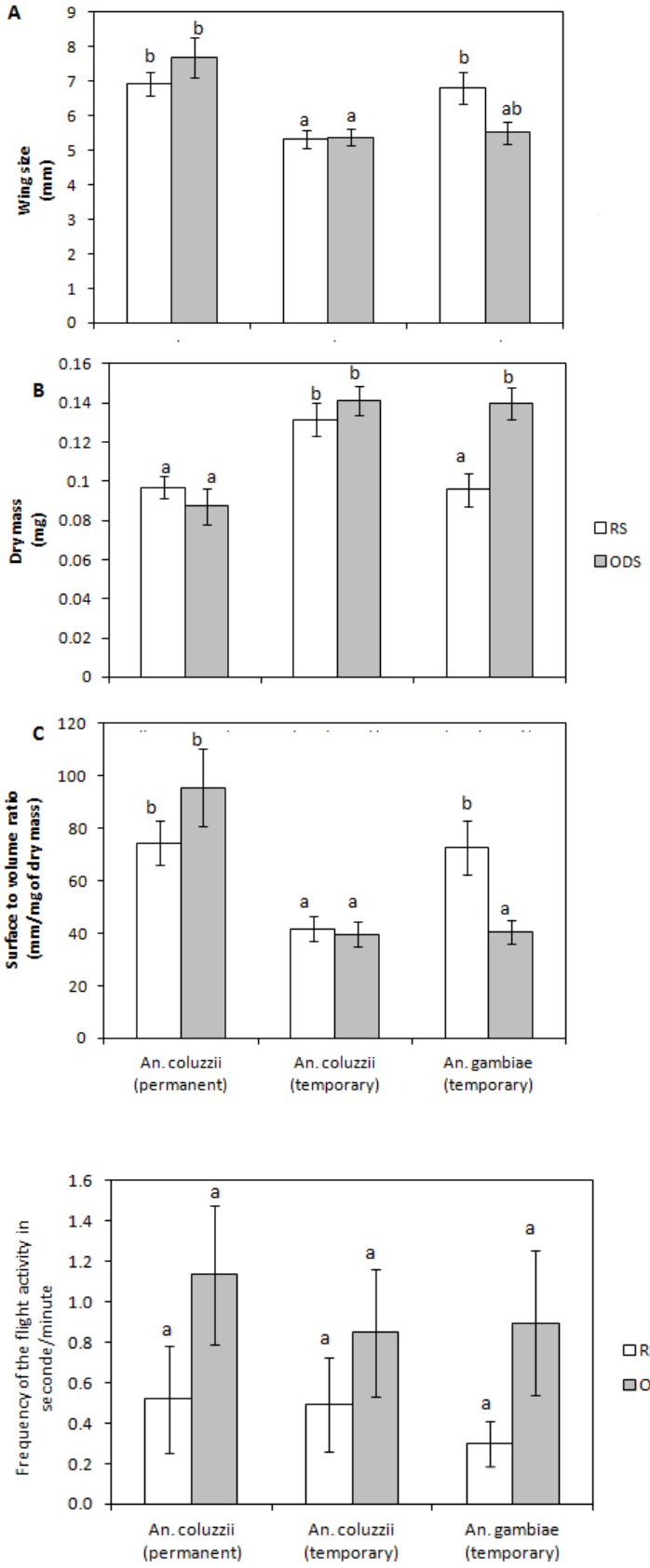

Fig. 2 Mean of wing size (A), female's dry mass (B) and surface to volume ratio (C), ±se in *An. coluzzii* (from both permanent and temporary breeding sites) and *An. gambiae* (from temporary breeding-sites) reared under RS (white bars) or ODS (dark grey bars) conditions. Different letter above bars indicate significant differences to α=0.05.

Fig. 3 Mean of the flight frequency activity (in s/min) ±se of *An. coluzzii* (from both permanent and temporary breeding sites) and *An. gambiae* (from temporary breeding-sites) reared under RS (white bars) or ODS (dark grey bars) conditions. Flight activity is represented Different letter above bars indicate significant differences to α=0.05.

3.2. Metabolic activity (VCO2)

The three populations displayed equivalent metabolic rates (GLM, *ddl*=2, χ^2= 0.15, *P*=0.46; **Table 2**). However, environmental conditions at which the mosquitoes were exposed to significantly influenced their metabolic rates (GLM, *ddl*=1, χ^2= 7.45, *P*<0.01; **Table 2; Fig. 4**). This last effect was due to females from the temporary population of *An. coluzzii*. Hence, the VCO2 of these females significantly increased when they were reared under ODS conditions (**Fig. 4**). Similar trend was observed in permanent population of *An. coluzzii*, but this trend was not significant. The wing size or the surface to volume ratio didn't significantly influence the volume of CO_2 released by females' mosquitoes (GLM, *ddl*=1, χ^2= 1.62, *P*=0.20; χ^2= 0.47, *P*=0.49; χ^2= 0.08, *P*=0.49, respectively; **Table 2**).

Table 2. Results of the GLM computed on the VCO2 with the experimental conditions, the anopheline populations, flight activity, surface to volume ratio and wing size as explicative variables. Signif. code: < 0.01 "**".

Effects	***ddl***	**χ^2**	***P*-value**
Environmental conditions	1	7.44	**
Population	2	1.54	0.46
Surface to volume ratio	1	0.08	0.77
Flight	1	1.62	0.20
Wing size	1	0.47	0.49
Environmental conditions : Population	2	5.11	0.07

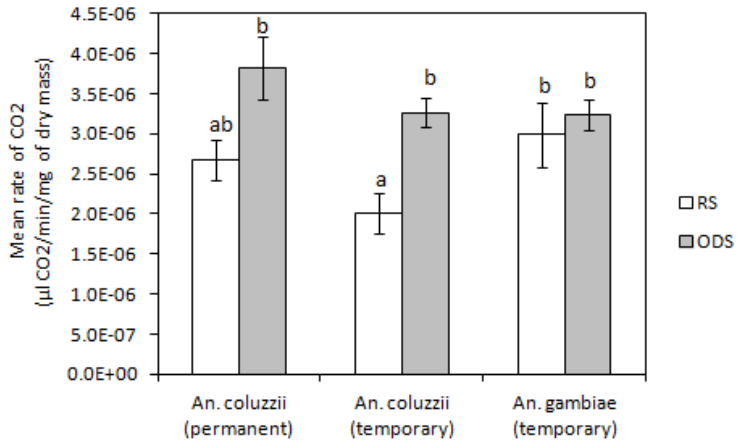

Fig. 4 Mean rate of CO2 ±se released by *An. coluzzii* (from both permanent and temporary breeding sites) and *An. gambiae* (from temporary breeding-sites) reared under RS (white bars) or ODS (dark grey bars) conditions. Different letter above bars indicate significant differences to α=0.05.

3.3. Body water transpiration (VH2O)

Overall, a significant effect of the population on VH2O was found (GLM, χ^2=7.96, *P*<0.05; **Table 3**), with *An. coluzzii* from temporary breeding sites displaying the lowest rate of water transpiration under RS conditions and permanent population of

An. coluzzii showing the highest rate in ODS conditions (**Fig. 5**). Moreover, VH2O significantly differed between the environmental conditions to which the mosquitoes were submitted (GLM, χ^2=76.97 *P*<0.001; **Table 3**), with all the populations showing a significant decrease in their rate of transpiration under ODS conditions. However, a significant interaction trend between environmental conditions and the population suggests that the impact of the environmental conditions on VH2O differed among the populations (GLM, χ^2=5.21, *P*=0.07; **Table 3**). This interaction was mainly supported by female *An. coluzzii* from permanent breeding site, which exhibited the greatest rate of H_2O release when exposed to ODS conditions (**Fig. 5**).

In addition, the surface to volume ratio of females significantly influenced the female transpiration (GLM, χ^2=91.04, *P*<0.001; **Table 3**). For all populations and for both environmental conditions, VH2O increased linearly with the surface to volume ratio, but this increase was greater for the mosquitoes submitted to RS conditions (**Fig. 6**), as supported by the significant interaction terms between surface to volume ratio and rearing conditions (GLM, χ^2=91.04, *P*<0.001; **Table 3**).

Table 3. Results of the GLM computed on the VH2O with the experimental conditions, the anopheline populations, VCO2, flight activity and surface to volume ratio as explicative variables. Signif. code: < 0.001 "***"; <0.05 "*".

Effects	***ddl***	χ^2	***P*-value**
Environmental conditions	1	76.97	***
Population	2	7.96	*
VCO2	1	0.39	0.53
Surface to volume ratio	1	91.04	***
Flight	1	0.05	0.82
Environmental conditions : Population	2	5.21	0.07
Environmental conditions : Surface to volume ratio	2	5.91	*

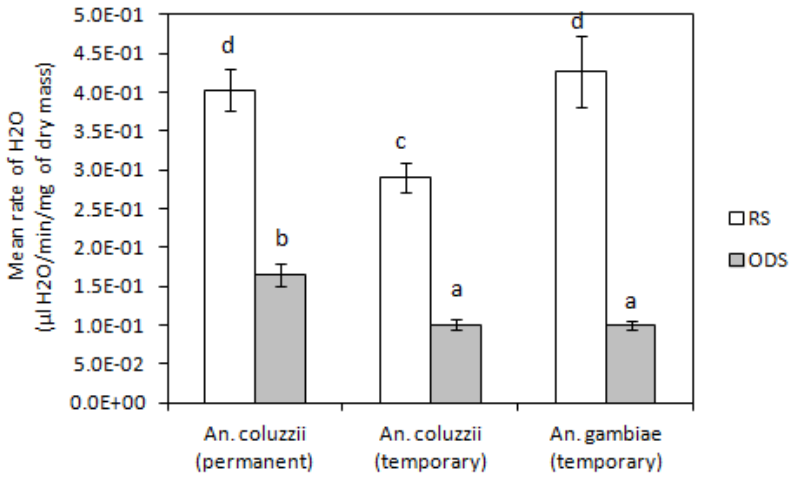

Fig. 5 Mean rate of H2O ±se released by *An. coluzzii* (from both permanent and temporary breeding sites) and *An. gambiae* (from temporary breeding-sites) reared under RS (white bars) or ODS (dark grey bars) conditions. Different letter above bars indicate significant differences to α=0.05.

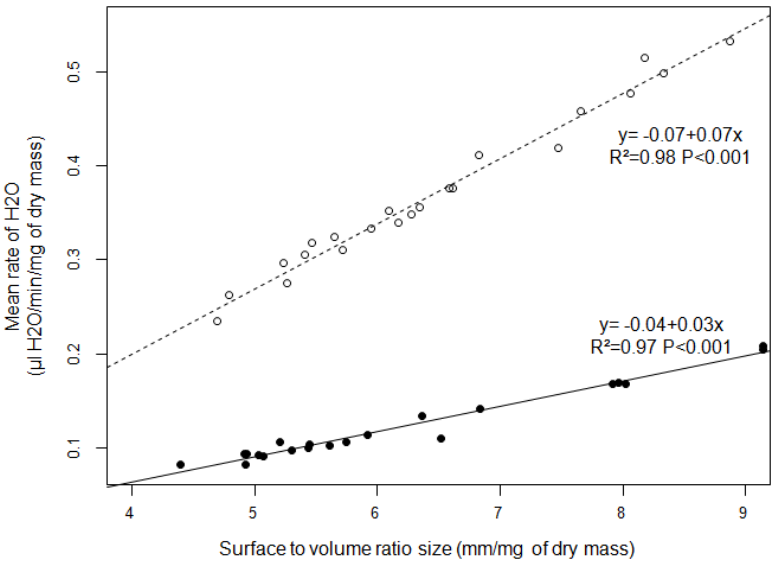

Fig. 6 Plot of the linear regression model performed between VH2O and the surface to volume ratio of female mosquitoes reared under RS (open circles, dashed line) or ODS (closed dark circles, solid line). Equations of the linear regression as well as the associated coefficient and probabilities are given above the lines.

3.4. Changes in *AKH*-related genes expression with environmental conditions

Expression levels of the *Anoga-AKH-R* and *Anoga-AKH-I* genes differed between the populations (ANOVA, ddl=2, $F_{AKH\text{-}R}$=5.95, $F_{AKH\text{-}I}$=2.80, P<0.05), but not those of *Anoga-AKH-II* (ANOVA, *ddl*=2, F=2.11, P=0.16). Expression of the three genes did not show a significant influence of environmental conditions but significant interaction term between environmental conditions and population was found (ANOVA, *ddl*=2, *Anoga-AKH-Receptor*: F=15.76, P<0.001; *Anoga-AKH-I*: F=8.33, P<0.01; *Anoga-AKH-II*: F=5.53, P<0.05). The significant interaction suggests that influence of the rearing environmental conditions on the three genes expression levels differed among the three populations (**Fig. 7**). Hence, *Anoga-AKH-R* and *Anoga-AKH-I* genes displayed strikingly similar patterns of expression, with a significant increase of their mRNA amounts between RS and ODS conditions for *An. coluzzii* from temporary breeding sites whereas a decrease is observed for *An. coluzzii* from temporary sites (**Fig. 7A-B**). A distinct pattern was noticed for the *Anoga-AKH-II* gene in which mRNA levels significantly increased for both populations from temporary sites whereas a decrease (although marginally significant) was observed for *An. coluzzii* from permanent breeding sites (**Fig. 7C**).

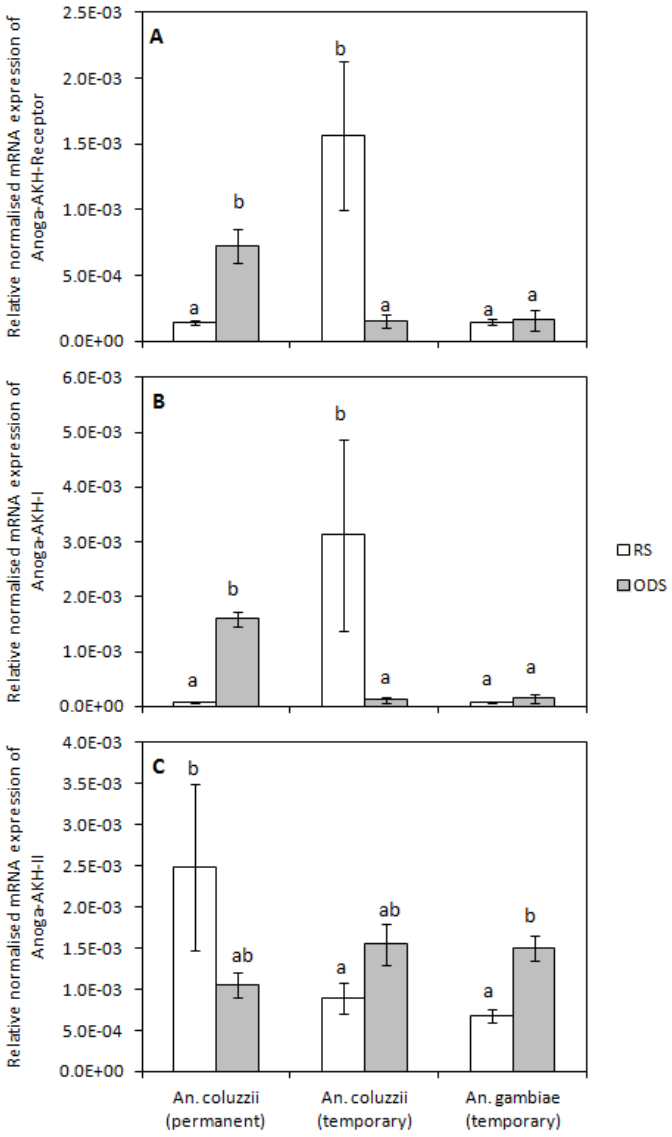

Fig. 7 AKH related gene expression differences between *An. coluzzii* from permanent and temporary breeding sites and *An. gambiae* reared under RS (white bars) and ODS (grey bars) environmental conditions. Data are presented as relative normalized amounts (±se) of mRNA of *Anoga-AKH-Receptor* (A), *Anoga-AKH-I* (B), and *Anoga-AKH-II* (C) genes. Different letters above the bars indicate significant differences to α=0.05.

4. DISCUSSION

Our study aimed to explore the influence of the environmental conditions of the onset of the dry season on several phenotypic traits of females *An. gambiae* and *An. coluzzii* sampled from distinct habitats where their population dynamics are contrasted ("permanent" *An. coluzzii* from sites where the populations are found breeding all year round, and "temporary" *An. coluzzii* from sites where the populations virtually disappear at the onset of the dry season). Our expectations were that the morphometry, the metabolic rate, the rate of H_2O release, the flight activity and expression levels of different AKH genes related to metabolite storage and mobilisation would display significant changes between the season and/or the different populations considered.

We awaited peculiar and specific phenotypes, as the three species/populations considered are supposed to display different survival strategies in the fields.

Like in previous studies, we used the carbon dioxide release by female mosquitoes (VCO2) to indirectly estimate their metabolic rate (Lighton & Fielden, 1995; Rourke, 2000; Terblanche *et al.*, 2005). VCO2 was measured on mosquitoes submitted to the conditions of temperature and relative humidity found during the hottest hours of the day in the field during the rainy season and the dry season. Hence, like in Huestis *et al.* (2012), our results give a "snap shot" of the metabolic rate of different species and populations of *An. coluzzii* and *An. gambiae* during the rainy season and the dry season. Generalising from studies on overwintering diapause (Hahn & Denlinger, 2007; Kambule *et al.*, 2011), we predicted that, when reared under dry season conditions, metabolic rate of mosquitoes would decrease in case of dormancy (*i.e.* in *An. coluzzii* strong or weak aestivator population) even when submitted to the hottest temperatures of the dry season, but would increase otherwise (in *An. gambiae*). In contrast to our expectations, metabolic rates of *An. coluzzii* from permanent and temporary breeding sites both showed a significant increase whereas no difference could be evidenced for *An. gambiae*. Nonetheless, these results are congruent with similar recent work performed on field *An. coluzzii* mosquitoes (Huestis *et al.*, 2012). Hence, our hypothesis of a decreased metabolic rate in these females is wrong, suggesting that the physiological coercions that insects have to face during aestivation might be different than those they face during overwintering diapause, which result in distinct mechanisms and VCO2 signatures (Denlinger & Armbruster, 2014). At the least, our results, like others (Huestis *et al.* 2012), emphasise such distinction between the physiological processes of aestivation in *An. coluzzii* and those of the overwintering diapause. However, we could not rule out the hypothesis that our environmental conditions and/or biological material weren't suitable to reproduce the diapause state. In this case, the increase in metabolic rate would be due to the fact that CO_2 emissions are sensitive to temperature changes, and there is evidence in the literature that an increase in temperature increases the metabolic rate (Clarke, 1993; Huestis *et al.*, 2012; Lardies *et al.*, 2004; Terblanche *et al.*, 2005). Indeed, depending on the temperature ranges the species are submitted and their physiological status (*i.e.* age, trophic and reproductive status, etc.), metabolic rate of insects can increase

linearly with ambient temperature (Nespolo *et al.*, 2003; Nielsen *et al.*, 1999; Rogowitz & Chappell, 2000; Rourke, 2000). For instance, the metabolic rate of *Chirodica chalcoptera* (Coleoptera: Chrysomelidae) is 1.5 times higher in individuals acclimated at 25 °C than those acclimated at 12 °C (Terblanche *et al.*, 2005). The high temperatures of the dry season might also promote an increase of mosquitoes' activity, and therefore their metabolic rates. A trend toward an increase of mosquitoes' flight activity emerges from our data, but this trend is not significant. In all cases, the fact that metabolic rate of *An. gambiae* mosquitoes is not affected by dry season conditions is of particular interest and highlights that this species puts in place distinct mechanisms than her counterparts to cope with the harsh environmental conditions.

One of the major benefices of decreasing metabolic rate for insects living in arid environments is to reduce water loss (Denlinger, 2002; Storey & Storey, 1990; Tanaka *et al.*, 1988). However, according to Chown (2002), depressing the metabolic rate doesn't lead by itself to a significant decrease in water losses, which is mainly achieved by adjustments of cuticular permeability (Bradley *et al.*, 1999; Djawdan *et al.*, 1997; Rourke, 2000; Williams *et al.*, 1998; Williams & Bradley, 1998). In accordance to these findings, our results also showed that metabolic rate variations did not play a significant role on water loss rates: an increase of VCO2 is not correlated with an increase in VH2O. On the contrary, mosquito's water loss drastically decreased in all populations under dry season conditions, which suggests that other mechanisms than adjustments of the metabolic rate reduce the transpiration of anopheline mosquitoes at the onset of the dry season.

The reduction of the surface to volume ratio is described in several species as one of the mechanisms preventing body water loss (Benoit & Denlinger, 2007; Hadley, 1994). Our data show clearly that a linear relationship exists between the rate of water losses and the surface to volume ratio in all the species/populations and for both environmental conditions. However, *An. gambiae* only shows a significant decrease of its ratio when reared under dry season conditions, suggesting this species is the only one susceptible to use this mechanism to reduce its water losses. In addition, we found that this decrease of the ratio is mainly due to an increase in the dry mass of *An. gambiae* females, highlighting potential differences in their abilities to harvest, store or use their metabolic resources at the onset of the dry season, like it was

shown for other species (Lounibos *et al.*, 2002; Reiskind & Zarrabi, 2012). Interestingly, linear relationships between water losses and surface to volume ratio differed between environmental conditions. If a significant linear relationship exists in both conditions, the same increase of the surface to volume ratio would lead to a smaller amount of water loss in ODS-reared mosquitoes than in RS ones. Hence, additional mechanisms might be triggered under these conditions to further minimise water losses.

For instance, changes in cuticle composition to increase its permeability might be involved (Chown, 2002; Chown & Nicolson, 2004; Hadley, 1994; Johnson & Gibbs, 2004). In this study, we did not explore such adjustments. Interestingly, changes in hydrocarbon quality and quantity, which are supposed to participate to the cuticle permeability, have been recently observed between seasons in *An. coluzzii* and *An. gambiae* (Wagoner *et al.*, 2014). Moreover, changes in aromatic amino acids (*i.e.* accumulation of phenylalanine, tryptophan and tyrosine) and proteins (*i.e.* accumulation of RR-2 cuticular proteins) composition have been also reported in both species, all these compounds being reported to participate to the sclerotisation process and to the increase of permeability of the cuticle (Article I; Goltsev *et al.*, 2009; Li & Denlinger, 2009; Truman & Riddiford, 1999).

Other changes including, the catabolism of carbohydrates like the stored glycogen may also participate to increase the desiccation resistance of mosquitoes (Gibbs, 2002; Gibbs *et al.*, 1997). Catabolism of glycogen is ensured by a glycogen phosphorylase which is regulated by adipokinetic peptides in insect species, including the *An. gambiae* (Article III; Gäde, 2004; Wilps & Gäde, 1990; Ziegler *et al.*, 2011). These peptides are well known for their role in sustaining flight activity through mobilisation of energetic resources in insects (Arrese & Soulages, 2010), but they are also suspected to enhance desiccation resistance as they allow the release and transport from the fat body to the hemolymph of organic compounds with osmoprotectants functions (*i.e.* trehalose, proline) in overwintering insects (Gäde, 2004; Hahn & Denlinger, 2011; Isabel *et al.*, 2005; Wilps & Gäde, 1990; Ziegler *et al.*, 2011). In this study, we measured the mRNA expression of two AKH peptide genes (*Anoga-AKH-I* and *Anoga-AKH-II*) and their putative receptor gene (*Anoga-AKH-Receptor*). Results first highlight that both *Anoga-AKH-I* and *Anoga-AKH-Receptor* genes show similar

pattern of variation in all female mosquitoes suggesting they form a specific receptor/peptide couple. By opposition, *Anoga-AKH-Receptor* variations did not match with those of the *Anoga-AKH-II*. Under ODS conditions, the AKH-I/AKH-Receptor couple was over-expressed in permanent population of *An. coluzzii* whereas it was under-expressed in the temporary one and did not show any variation in *An. gambiae*. The *Anoga-AKH-II* gene showed the same increase in its expression level in both temporary populations but was under-expressed in *An. coluzzii* permanent population. As we expected, both genes show distinct patterns of expression between populations/species and between environmental conditions, with a rather clear distinction between the permanent population and the temporary ones. This highlights the fact that both genes might have different functions, although they are still unknown (Kaufmann & Brown, 2006, 2008). Moreover, it seems obvious that these two AKH peptides are involved in the mechanisms put in place by Anopheles species to survive to the dry season and, as such, could serve as physiological markers of their peculiar strategies. In particular, our results suggest the AKH-1 gene expression characterised the distinct responses of "permanent" populations of mosquitoes whereas the AKH-II seems specific to the "temporary" ones.

5. CONCLUSION

Our study shows that female mosquito ***An.*** *gambiae* exhibit constant and elevated metabolic rate throughout the year, whereas female *An. coluzzii* exhibit seasonal variations. However, the seasonal variations in metabolic rate observed in *An. coluzzii* are not in accord to our primary hypothesis of an aestivation strategy in these females at the onset of the dry season. Indeed, our hypothesis was based on the physiological processes known for the overwintering diapause, but both aestivation and winter diapause may involve distinct physiological processes. Understanding the mechanisms of aestivation in *An. coluzzii* and other tropical species thus remains a major challenge (Denlinger & Armbruster, 2014; Yaro *et al.*, 2012). In addition, we show that metabolic rate of mosquitoes do not impact the mosquito's transpiration. Rather, changes in surface to volume ratio in *An. gambiae* play a significant role in decreasing water losses. Finally, our results

evidence interesting pattern of seasonal variations in AKH peptides expressions. In particular, under ODS conditions, permanent population of *An. coluzzii* over-expressed the Anoga-AKH-I, whereas temporary populations of *An. coluzzii* and *An. gambiae* over-expressed the Anoga-AKH-II. Further investigations are required to examine the exact roles of these two peptides in the mosquito metabolism, but we believe they could constitute relevant marker of the specific dry season strategies developed by "permanent" and "temporary" anopheline populations during the dry season.

Acknowledgements

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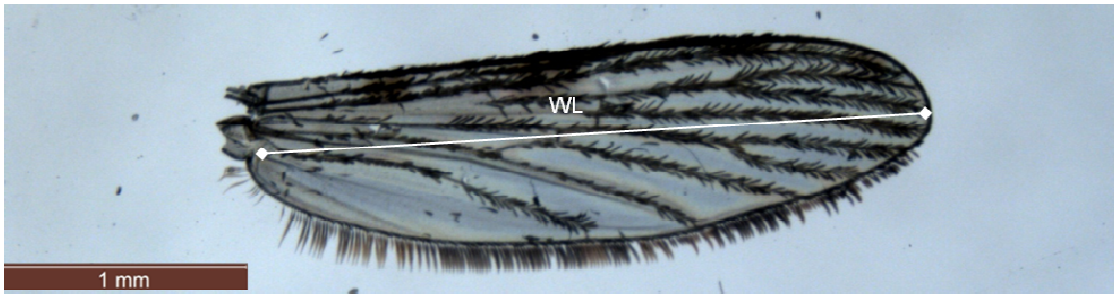
SUPPLEMENTARY DATA



Supplementary data 1. Position of the 2 landmarks used to measure the lengths of female wings (WL).

Chapitre III

Variabilité de la plasticité phénotypique chez *An. coluzzii*

1. **Variabilité de la plasticité physiologique des femelles *An. coluzzii*** (Article III)

2. **Temps de développement pré-imaginal et plasticité morphologique chez *An. coluzzii*** (Article IV)

1. Variabilité de la plasticité physiologique des femelles *An. coluzzii* (Article III)

Dans le chapitre précédent, nous avons mis en évidence une variabilité des réponses des populations d'*An. coluzzii* aux conditions déshydratantes de la saison sèche selon que les femelles exploitent des gîtes larvaires permanents ou uniquement présents pendant la saison des pluies (Article II). Des différences au niveau de l'allométrie et de l'expression des hormones adipokinétiques (AKH) ont été observées selon les conditions d'élevage des femelles mais également selon leur origine géographique (Article II). Ces résultats suggèrent que la disponibilité des points d'eau dans l'habitat exerce une forte pression locale sur les moustiques et induit des mécanismes de survie différents entre les populations d'*An. coluzzii*. Il a notamment été suggéré que les femelles *An. coluzzii* ont différentes capacités d'estivation selon la présence d'eau dans l'habitat. D'après Yaro *et al.* (2012), les femelles *An. coluzzii* entrent dans un état de dormance estivale profond lorsque l'habitat ne dispose pas de points d'eau pendant la saison sèche, ou dans un état de dormance estival peu prononcé (quiescence) lorsque les points d'eau sont encore disponibles, permettant la reproduction et la ponte de la nouvelle génération. Si ces hypothèses sont vérifiées, alors des différences au niveau du métabolisme (ex. stockage et utilisation des réserves, métabolisme énergétique, etc.) des insectes devraient être mises en évidence chez les femelles *An. coluzzii*, notamment à l'approche de la saison sèche. L'objectif de ce troisième travail est donc d'évaluer la variation de la plasticité physiologique des femelles *An. coluzzii* en réponse à la saison sèche selon l'origine géographique des populations.

Quatre populations de femelles *An. coluzzii* échantillonnées au nord et au sud du Burkina-Faso dans des gites larvaires (points d'eau) permanents ou seulement présents pendant la saison des pluies ont été utilisées dans le cadre de ce travail. Au Burkina-Faso, les conditions de pluviométrie annuelle et la durée de la saison sèche varient selon un gradient latitudinal : les populations du nord font face à une saison sèche plus drastique qu'au sud. Nous supposons donc que la disponibilité des points d'eau dans l'habitat pendant la saison sèche combinée à la sévérité des conditions de cette saison doit influencer les mécanismes de survie des femelles. Par exemple, la disparition des points d'eau dans l'habitat au nord du Burkina-Faso devrait induire des

réponses plus marquées qu'au sud. Les femelles *An. coluzzii* devraient donc montrer des ajustements physiologiques distincts en fonction de la disponibilité en eau dans leur habitat (permanent *versus* temporaire) et selon leur origine géographique (nord *versus* sud).

Nous avons analysé et comparé un grand nombre de paramètres physiologiques dans le but d'identifier des marqueurs de réponses à la saison sèche spécifiques aux différentes populations**.** Le choix des réserves corporelles catabolisées par les insectes renseigne sur les besoins énergétiques de ces derniers. Nous avons donc tout d'abord analysé les variations des teneurs en protéines, triglycérides et l'expression moléculaire de la glycogène phosphorylase et de la glycogène synthase, deux enzymes impliquées dans le métabolisme du glycogène. Ces dosages ont été réalisés dans chaque population d'*An. coluzzii* exposées à des conditions reproduisant la saison des pluies (RS) ou le début de la saison sèche (ODS). Nous avons ensuite analysé les profils métaboliques des femelles de chaque population exposées aux conditions RS et ODS. Cette analyse permettra de mettre en avant des métabolites impliqués dans les mécanismes de survie des femelles de chaque population (ex. synthèse de polyols ou acides aminées osmoprotecteurs, intermédiaires du cycle de Krebs, etc.). Nous supposons que des profils différents entre populations devraient être observés. Enfin, la régulation du métabolisme énergétique de ces femelles a été analysé au travers l'expression des gènes codants les AKH. Des différences entre populations issues de gîtes permanents et temporaires ont déjà été mises en évidence (Article II). Nous souhaitons vérifier ces résultats et les généraliser aux populations du nord et du sud, dans le but de tester si l'expression des AKH constitue un marqueur des réponses des femelles *An. coluzzii* aux conditions de la saison sèche selon la disponibilité des points d'eau dans l'habitat.

Physiological plasticity of water stress resistance between geographic populations of the malarial mosquito *An. coluzzii* – (Article III)

Hidalgo, K., Siaussat, D., Braman, V., Mouline, K., Simard, S., Renault, D.

In pep for Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology

Abstract

Phenotypic plasticity allows adaptation/acclimation of insects to temporal and/or spatial varying environmental conditions. In Sub-Saharan Africa, insect populations, including the malarial mosquito *Anopheles coluzzii*, are periodically exposed to desiccating conditions (*i.e.* dry season) of which the severity depends on the water ponds availability in their habitat. In this context, mosquito populations may have developed distinct physiological responses to optimise its Darwinian fitness and locally persist during the dry season. The aim of this work is therefore to evaluate the range of physiological mechanisms elicited by distinct geographical populations of *An. coluzzii*, at the onset of the dry season. We assumed that changes in the amounts of protein, triglyceride, glycogen and circulating metabolites, and in the expression level of AKH peptides should elicited by mosquitoes to survive the dry season, but the nature and directions of these changes should also differ among the different populations. Four *An. coluzzii* populations, sampled at the North and South of the Burkina-Faso, in habitats characterised by permanent or temporary water ponds, were thus used to test our hypotheses. Significant differences were found between populations from permanent and temporary water ponds in the amount of circulating metabolites and in the expression level of AKH peptides. In particular, populations issued from temporary water ponds exhibit increased levels of AKH peptides and circulating metabolites with osmoprotectant functions (*e.g.* inositol, proline), suggesting high water stress in these mosquitoes. Changes in the amount of stored body reserves are also observed among population. Overall, our results suggest that the habitat characteristics (*i.e.* water ponds availability) exert a strong influence on the physiological responses of mosquitoes to elicit the desiccating conditions of the dry season.

Key words: glycogen; body stores; AKH peptides; water stress; compatible solutes; *Anopheles gambiae s.l.*

List of abbreviations: RS = rainy season; ODS = onset of the dry season; RH = Relative Humidity; LDA = Linear Discriminant Analysis; LD1 = First axis of the Linear Discriminant Analysis ; LD2 = Second axis of the Linear Discriminant Analysis

	North		**South**	
	Déou	Oursi	Soumousso	Bama
	Temporary	Permanent	Temporary	Permanent
Protein	↘	↘	-	-
Triglycéride	-	-	↗	-
Glycogen synthase	↘	-	↘	-
Glycogen phosphorylase	-	-	↗	↗
AKH-I	↗	-	-	-
AKH-II	-	-	↗	-

Graphical abstract Resume of the main physiological adjustments found in the four *An. coluzzii* populations when they exposed to ODS conditions.

1. Introduction

Environmental conditions, and more particularly thermal and relative humidity characteristics, play major roles in determining the spatial and temporal distribution of many terrestrial insects (Benoit *et al.*, 2010; Chown, 1993; Chown & Nicolson, 2004; Hoffmann & Harshman, 1999). In several habitats, thermal and desiccation stresses occur concomitantly, and there are supporting evidence that the deleterious effects of desiccating conditions may be compounded with heat exposures. In addition, the range of temperature and relative humidity encountered by any given species often vary across its geographical range, resulting in the expression of a number of distinct phenotypes among populations sampled from different habitats (*i.e.* several genotype/environment interactions, building reaction norms; Schlichting & Pigliucci, 1998). Trait flexibility thus represents an essential strategy of the species allowing acclimation to varying conditions at different time and geographical scales, which in turn enables permanent optimisation of the Darwinian fitness (Ghalambor *et al.*, 2007). Moreover, at the local scale, the continued selection pressure exerted by the environmental factors may lead to local adaptation (see Kawecki & Ebert, 2004 for a review), with populations of a given species exhibiting increased fitness in their local habitats as compared to specimens from other habitats.

Several mechanisms occurring at different organisational levels have been developed by insects to face variations in temperature and relative humidity in their natural habitat (Chown *et al.*, 2010, 2011; Denlinger & Yocum, 1998; Neven, 2000; Michaud *et al.*, 2008; Thorat *et al.*, 2012), including increased body water storage (metabolic water stores, bulk water), reduced body water loss rates or increased tolerance to body water loss (Benoit, 2010; Danks, 2000). The mechanistic bases of these strategies are highly physiologically-based, and it is for instance assumed that stored glycogen permits significantly higher amount of bounded water as the stored lipids of fat body does (Schmidt-Nielsen, 1990). It is also interesting mentioning that glycogen degradation can release up to 5 times its mass in water molecules (Gibbs, 2002; Gibbs *et al.*, 1997; Schmidt-Nielsen, 1990) and that glycogen can provide precursors for synthesis of osmoprotectants in the form of sugars, polyols or amino

acids (Danks, 1987; Hahn & Denlinger, 2007; Steele & Paul, 1985). Among these, the trehalose and the proline are common osmoprotectants accumulated in insects facing water stress and desiccation (Rudolph & Crowe, 1985; Thorat *et al.*, 2012; Yancey, 2005), as demonstrated in larvae of the mosquito *Culex tarsalis* (Diptera, Culicidae) (Patrick & Bradley, 2000). Low molecular weight sugar alcohols such as sorbitol and inositol can also be used by insects to limit damages induced by water stress on membranes and proteins (Yancey, 2005). Considering the central role that could be played by glycogen in enhancing desiccation resistance in insects, metabolic shifts are thus expected in specimens that experience dry conditions in their habitats. Individuals should thus exhibit increased glycogen deposits and catabolism in response to exposure to desiccating conditions.

Glycogen metabolism chiefly involves two enzymes, glycogen synthase and glycogen phosphorylase, the activity of which was shown to vary in water-stressed nematodes (Gal *et al.*, 2001) and collembolans (Worland *et al.*, 1998). Moreover, there is evidence that the activity of the glycogen phosphorylase is controlled by the adipokinetic hormones (AKH) (Gäde, 2004; Wilps & Gäde, 1990; Ziegler *et al.*, 2011). For instance, in *Manduca sexta* (Lepidoptera, Sphingidae) larvae, the release of AKH peptides results in increased activity of the glycogen phosphorylase when specimens were exposed to heat stress (*i.e.,* 35 °C) (Arrese & Soulages, 2010). AKH peptides are also suspected to prompt the release and transport of osmoprotectants (*i.e.,* trehalose, proline) from the fat body to the hemolymph in stressed insects (Gäde, 2004; Isabel *et al.*, 2005; Wilps and Gäde, 1990; Ziegler *et al.*, 2011). Changes in AKH peptide expression are thus expected in organisms facing desiccating conditions because AKH peptides are also involve in the control of the fuelling of the intermediary metabolism and in the tight regulation of aerobic metabolism in insect entering in diapause (Hahn & Denlinger, 2011; Isabel *et al.*, 2005; Kodrík *et al.*, 2003; Lee & Park, 2004; Woodring *et al.*, 2002).

In the present study, we explored physiological plasticity in response to water stress in population of the malarial mosquito, *Anopheles coluzzii* (Diptera, Culicidae). Female mosquitoes were exposed to contrasting conditions mimicking the environmental conditions they experience in their natural habitat in Burkina-Faso

(West Africa) during the rainy season and at the onset of the dry season. In this country, annual rainfall and duration of the dry season vary according to a latitudinal gradient, with northernmost regions exhibiting low annual rainfalls and a long dry season (*i.e.* >7 months). Populations of *An. coluzzii* can be found in both permanent and temporary breeding-sites (Baldet *et al.*, 2003; Gimonneau *et al.*, 2012). Hence, in areas where large anthropogenic (*e.g.* dams, ricefields, etc.) or natural (*e.g.* ponds, rivers edges, etc.) surface water collections are yearly available, this mosquito can breed all year-long (*i.e.* hereafter referred to as permanent population in the text). Conversely, in areas where surface water is available only during the rainy season, populations locally disappear during the dry season (*i.e.* hereafter referred to as temporary population in the text), suggesting that different populations of this species may have developed distinct seasonal phenotypes to resist the desiccating conditions of the dry season (Article IV; Yaro *et al.*, 2012).

To conduct this study we used four distinct geographic populations of *An. coluzzi* from the Burkina-Faso, sampled from a latitudinal gradient in both permanent and temporary breeding-sites. We expected that the physiological plasticity to water stress would vary according to the geographic origin of the mosquitoes as a result of local adaptation/acclimation. In particular, we hypothesised that exposure to dry conditions would trigger changes in whole body reserve levels resulting in glycogen and increased amounts of circulating metabolites with osmoprotectant roles. Increase expression of AKH peptides would also observed as they suppose to participate in increase glycogen phosphorylase activity and mobilisation of osmoprotectant metabolites. We also assumed that specimens from locations where water collections are temporary should be characterised by "strong aestivator" phenotypes programmed to engage into a state of diapause (see Yaro *et al.*, 2012). Based on our knowledge on the overwintering diapause, we supposed these mosquitoes would exhibit increase degradation of glycogen, high amount of lipid and osmoprotectants, and increase expression of AKH when exposed to dry conditions.

2. Materials & Methods

2.1. Sample mosquito

Experiments were conducted using four mosquito populations derived from the progeny of wild-caught *An. coluzzii* females sampled from the North (N = 2) and South (N = 2) regions of the Burkina-Faso (**Fig. 1**). In Burkina-Faso, wild-caught female mosquitoes were sampled from (i) anthropogenic breeding-sites with permanent surface waters (*i.e.* presence of year-round mosquito breeding opportunities in Oursi [14°40'N, 0°27'W] and Bama [11°23'N, 4°24'W]), and (ii) natural breeding-sites where surface waters disappear at the onset of the dry season (*i.e.* Déou [14°35'N, 0°43'W] and Soumousso [11°04'N, 4°03'W]). The populations were further maintained under controlled conditions (27 ± 1°C, 80 ± 10% relative humidity with LD cycles of 12h:12h) at the IRSS (Institut de Recherche en Science de la Santé) of Bobo-Dioulasso for six generations, prior to being used for experiments.

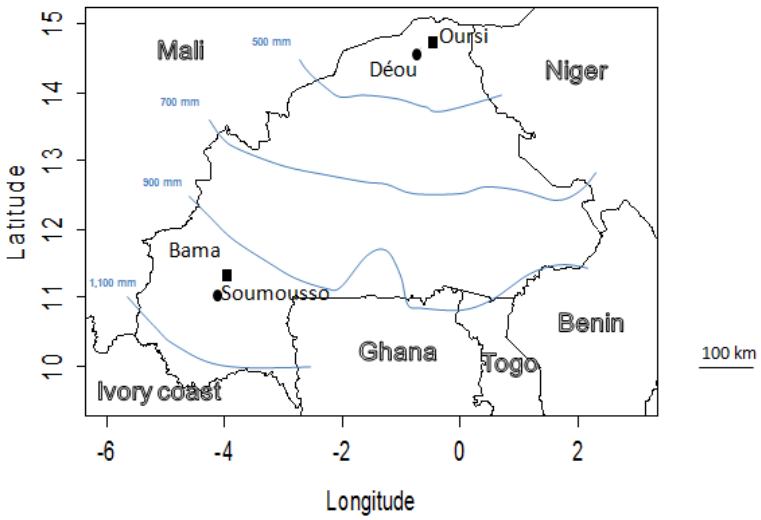


Fig. 1 Geographical localisation of the four localities where wild *An. coluzzii* mosquitoes were collected in northern and south-western regions of Burkina-Faso (West Africa). Squares represent permanent mosquito populations and circles represent temporary populations (see text). The blue lines represent the annual isohyete (in mm) at the Burkina-Faso.

2.2. Experimental conditions and mosquito rearing

The four anopheline populations were reared from eggs to adults in programmable climatic chambers (Sanyo MLR 315H, Sanyo Electric Co., Osaka, Japan). The chambers reproduced the climatic conditions (*i.e.* temperature, relative humidity (RH), and photoperiod) recorded in Oursi (the northernmost region of Burkina-Faso) during the rainy and the onset of the dry season. Hourly temperature and RH, and photoperiod

duration were set up using the climatic data available at http://www.gaisma.com/ for the Oursi area, averaged over the past 30 years for a period ranging from August 1st to 31th (*i.e.* rainy season, hereafter referred to as RS conditions; N=1 climatic chamber) and from December 1st to 31th (*i.e.* at the onset of the dry season, hereafter referred to as ODS conditions; N=2 climatic chamber). Climatic conditions were hourly average and a twelve-step cycle was designed inside the climatic chambers to reproduce as closely as possible the natural daily climatic fluctuation of RS and ODS conditions. MicroLog Pro monitors (EC750, Davis Instruments, Hayward, CA, USA) were used to monitor temperature and RH inside the incubators and inside one of the rearing pans to monitor larval rearing water temperature (see **Fig. 2**).

For each mosquito population, eggs were collected from three to four independent rearing sessions to reach the sample size needed to conduct our experiments. Each session consisted of two batches of eggs from more than fifty females. Eggs were merged to achieve large sample sizes and synchronous hatching. The programmable RS and ODS conditions inside the three climatic chambers were alternated among the different rearing sessions. Upon collection, eggs were directly transferred into independent plastic trays (30 cm × 20.5 cm × 6.5 cm), each containing 1 L of deionised water. Trays containing eggs were immediately transferred to the RS or ODS experimental conditions within the climatic chambers. After hatching, first instar larvae were readily transferred into new plastic trays filled with 1 L of deionised water at an optimal growing density of about two hundred larvae per tray. For each session, two to three plastic trays (*i.e.* about 400–600 larvae) were used for each mosquito population and each experimental condition (RS, ODS). Every day, the position of the trays was randomly alternated to avoid any positional effect within the incubator. Larvae were fed daily with sprinkled ground fish food (Tetramin®) provided *ad libitum* until pupation. Pupae were collected and immediately transferred into new plastic cups (diameter 7 cm × height 8.5 cm) filled with 10 mL of deionised water, and maintained under RS or ODS conditions until adult emergence. Soon (<1 h) after emergence, males were discarded, and females were kept into new plastic cups with access to a 10% glucose solution *ad libitum*. Females were maintained into the climatic chamber in their respective growing conditions for a maximum of seven days

(± 1 hour). Note that females were deprived from food and water 24 hours before the experiments in order to avoid biasing physiological measurements due to the presence of aliments with differential filling of the gut.

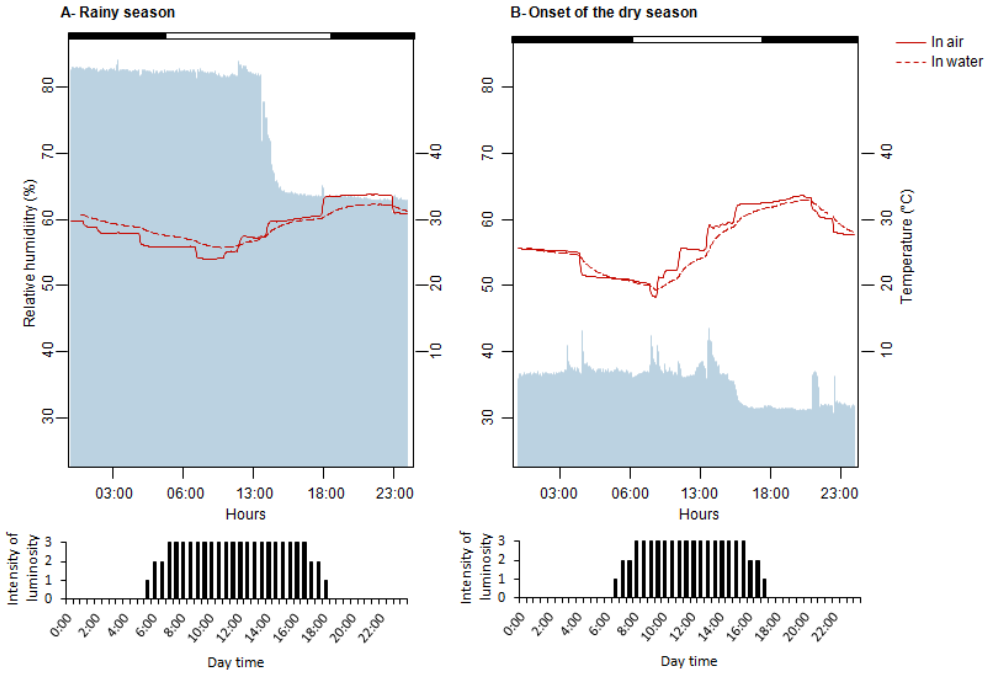


Fig. 2 Daily fluctuations of RH (%; blue bars) and temperature (°C, red lines) in the climatic chambers (solid lines) and within rearing trays filled with 1 L of deionised water (dashed lines) during the rainy season (A), and at the onset of the dry season (B). The meteorological conditions recorded from Oursi were used. For each seasonal condition, photoperiod duration is represented by the black bars below.

2.3. Body water content and dry mass

For each population and each experimental condition (RS, ODS), fresh masses of newly emerged (<1h-old specimens, hereafter referred to as T0), two-days old (hereafter referred to as T2), and seven-days old (hereafter referred to as T7) females were measured using a microbalance (Sartorius SE2, d=1µg). Specimens were then dried for three days at 60 °C before being reweighed (dry mass). Body water content was determined for each female as the subtraction between fresh and dry masses, and expressed in mg per mg of dry mass. A total of 1,056 female mosquitoes randomly chosen from the different rearing sessions were used to conduct this experiment. After insured that no rearing session effect existed, females were analysed independently of the rearing session they issued.

2.4. Body stores

2.4.1. Protein and triglyceride contents

For each population and experimental condition, five-to-six replicates, each consisting of a pool of four-to-five T7 females (ensuring a minimum sample dry mass of 1 mg) maintained in their respective growing conditions were collected and immediately snap-frozen. Each pool of mosquitoes was freeze-dried (Lyovac™ GT3) for 48 h before measuring dry mass (d = 1µg, Mettler Toledo GmbH©, Greinfense, Switzerland). Like for body water and dry mass, females used to conduct these assesses were randomly chosen from the different rearing sessions, then combined after assuring that no rearing session effect was observed.

2.4.2. Protein extraction and quantification

The total of protein content was assessed using a Sigma protein assay kit (Sigma Chemical Co., BCA-1), following the procedure described by Bradford (1976) and revisited by Foray *et al.* (2012). Protein were extracted in 180 µL of phosphate buffer (100 mM KH_2PO_4, 1 mM DTT and 1 mM EDTA, pH 7), and homogenised with a bead-beating at 30 Hz for 1.5 min (Retsch™ MM301, Retsch GbmH, Haan, Germany). The concentration of total proteins was read at 595 nm (Molecular Devices VERSAmax Tunable) following the manufacturer's instructions. Whole body protein contents were expressed in nmoles/mg of pool sample dry mass.

2.4.3. Triglyceride extraction and quantification

A 600 µL volume of methanol-chloroform (1:2, v:v) solution was added to each pool of four-to-five T7 females. The samples were further homogenised using two 3 mm tungsten beads at 30 Hz for 1.5 min (Retsch™ MM301, Retsch GbmH, Haan, Germany). Then, a volume of 200 µL of ultrapure water was added (final methanol-chloroform-water solution 1:2:1, v:v:v). The samples were homogenised and centrifuged at 4,000 g for 10 min at 4 °C. A 300 µL aliquot of the lower chloroform-lipidic phase was transferred into a clean 1.5 mL microtube, and dried out at 25 °C. The dry residues were then re-suspended in 100 µL of BSA-Triton X100-water solution (0.3:0.02:1, v:v:v), as described in Laparie *et al.* (2012). Samples were vortexed and incubated for 10 min at 60 °C. Triglyceride contents were measured

using a colorimetric assay kit (Triglyceride assay kit, Cayman Chemical Company, Ann Arbor, MI, USA), following the manufacturer's instructions. Absorbance was read at 510 nm (Molecular Devices VERSAmax Tunable) and triglyceride contents were expressed in nmoles/mg of mosquito dry mass.

2.5. Expression of Glycogen synthase and phosphorylase and Anoga-AKHs genes

2.5.1. RNA extraction and cDNA synthesis

For each population and experimental condition (RS, ODS), four replicates were used, each replicate consisting of pooled of ten females collected at T7 from the different rearing session, then combined after assuring there was no rearing session effect. Pools of ten females were immediately snap-frozen after collection and stored at -80 °C until being used for the analyses. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) coupled with the RNeasy Kit (QIAGEN), before being treated with DNase I (Ambion, USA) in accordance with the manufacturer's instructions. RNA amount was quantified by spectrophotometry at 260 nm (Nanodrop2000, Thermo Scientific). Single-stranded cDNAs were then synthesised from total RNAs with Superscript II reverse transcriptase (Gibco BRL, Invitrogen) as described in Bigot *et al.* (2012) and according to the manufacturer's instructions.

2.5.2. Real-time quantitative PCRs

All Real-time quantitative PCRs were conducted as described in Bigot *et al.* (2012). A total of ten genes (*Actine*, *Rps13*, *Rps7*, *Rpl5*, *h3a, Cytp450, Tubulin, hsp83*, *EGFR*, and *18s*) were tested as putative housekeeping genes. Following a BestKeeper analysis (Pfaffl *et al.*, 2004), the Rpl13 was selected as the reference gene since its expression was stable in all samples whatever the experimental condition or anopheline population tested. Further, mRNA of the *glycogen synthase* and *glycogen phosphorylase* and the two AKHs genes *Anoga-AKH-I* and *Anoga-AKH-II* (see Kaufmann & Brown, 2006) were examined. Specific primers (reverse and forward) for both housekeeping genes and target genes were designed using the Eprimer3 software (http://emboss.bioinformatics.nl/cgi-bin/emboss/eprimer3; **Supplementary data 1**).

Each PCR reaction was technically triplicated and consisted of 6 µL of absolute Blue SYBR Green Fluor (ROCHE Molecular Systems Inc., USA), 2 µL of

cDNA (25 ng.µl^{-1}), 0.5 µL of each reverse and forward primers (10 µM), and 3 µL of RNA free water. The qPCR program and conditions were as in Bigot *et al.* (2012). The cycle threshold values (Ct-values) for both reference and target genes were determined using the Light-Cycler® 480 software (Roche, France). The average Ct value of each technical triplicate was used to normalise candidate gene expression levels to the geometric mean of the reference gene level using the Q-Gene software (Simon, 2003).

2.6. Metabolic profiling

The metabolic profiles were measured from the whole body of seven-days old (T7) female mosquitoes reared under RS or ODS conditions and randomly chosen in the different rearing sessions, then combined after assuring no effect of the rearing session. Extractions were conducted on pools of four females (N = 9 replicates for each experimental condition), so that the minimum sample dry mass was 1 mg. Upon collection, pools of T7 females were immediately snap-frozen in liquid nitrogen, and freeze-dried for 48 hours. Dry masses from each sample were then measured using a micro-balance (d = 1µg, Mettler Toledo GmbH©, Greinfense, Switzerland).

The samples were homogenised in 300 µL of methanol-chloroform (2:1, v:v) using a bead beating device (two tungsten beads of 3 mm) at 25 Hz for 1.5 min (Retsch^{TM} MM301, Retsch GbmH, Haan, Germany). A 10 µL volume of arabinose (3 mM, internal standard) was added to each sample to monitor the reliability of the metabolite quantification. Then, 200 µL of ultrapure water was added, and the samples were homogenised. Samples were centrifuged at 8,000 g for 5 min at 4 °C. A 150 µL aliquot of the upper aqueous phase, which contained polar metabolites, was transferred into a clean microtube, and vacuum-dried at 30 °C (Speed Vac Concentrator, MiVac, Genevac Ltd., Ipswich, England). The polar phase aliquots were re-suspended in 30 µL of freshly prepared methoxyamin hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine (20 mg/mL) prior to incubation under orbital shaking at 30 °C for 60 min. Following incubation, a 30 µL volume of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added, and derivatisation was conducted at 37 °C for 30 min.

A gas chromatograph-mass spectrometer (GC-MS) platform (Thermo Fischer Scientific Inc, Waltham, MA) was used for metabolite quantification. We used the

temperature ramping, mass spectrometer settings and chromatogram annotation described in Article VI. All samples were run under the SIM mode (electron energy: -70 eV) with a 30 m fused silica column (TR-5MS, 95 % 200 dimethyl siloxane, 5 % Phenyl Polysilphenylene-siloxane, I.D.: 0.25 mm). One microliter of each sample was injected using the split mode (25:1). Randomised sample sequences were established for sample injection, and chromatograms were deconvoluted using *XCalibur* v2.0.7. Standard samples, consisting of 61 pure reference compounds at 1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000, 1500, and 2000 µM were run and metabolite levels were quantified using the quadratic calibration curves for each reference compound.

2.7. Data analysis

All statistical procedures were conducted with the *R* 3.1.1 statistical software (R Development Core Team, 2008). Before analysis, the normal distribution (Shapiro-Wilk test) and homoscedasticity (Bartlett and Hartley tests) of the data were checked.

To explore the variation in body water contents, an ANCOVA model was performed using the two experimental conditions (RS, ODS), the four anopheline populations, and the female's age (T0, T2, and T7) as explanatory variables, and the dry mass of the female as explanatory covariable. Main variable effects and all relevant first and second order interactions were tested in full models. Further, model simplification used stepwise removal of terms, where the significance of the terms was estimated using the difference in Akaike's information criterion (AIC). Statistical significance was set at α=0.05 and Tukey HSD procedures were used in order to perform *post-hoc* comparisons among the levels of significant factors, when required.

Variations in females dry mass was then examined using an ANOVA performed with the two experimental conditions (RS, ODS) and the four anopheline populations as explanatory variables. Similar procedures were used to examine variation in body protein and triglyceride contents, and expressions level of *glycogen synthase*, *glycogen phosphorylase* and *Anoga-AKHs* genes.

A multivariate discriminant analysis was used to assess differences in the metabolite contents between the two experimental conditions and among the four anopheline populations. Metabolite contents were first log-transformed ($x = \log_{10}[X + 1]$) to fulfil the assumption of normally distributed residuals. Using the log-

transformed data, we performed a MANOVA to address physiological differences among the ten experimental groups. The class separation was further investigated using a linear discriminant analysis (LDA). Together with the multivariate analysis, two-way ANOVAs were performed for each detected metabolite, with experimental conditions and anopheline populations as explanatory variables. ANOVAs were followed, when required, by Tukey HSD *post-hoc* procedures among levels of significant factors. The statistical *P*-values were adjusted using the two-stage Benjamini-Hochberg algorithm (Benjamini & Hochberg, 1995) to control for false discovery rate (α=0.05) induced by multiple comparisons. Metabolites for which no significant difference could be demonstrated between at least two experimental groups (*i.e.* RS *versus* ODS; 4 populations) were discarded from the discriminant analysis to highlight the main metabolites contributing to physiological differences among the experimental groups. Likewise, samples encompassing metabolites that were not reliably quantified (signal/noise < 10, or concentration < quantification limit) were also discarded from the analysis. The between- and within-group degrees of freedom together with the F-value are reported for each LDA axis. Distribution probability of the LDA was also monitored using a Monte-Carlo permutation (10,000 random permutations; *P*-value<0.001).

3. Results

3.1. Body water content and dry mass

For each mosquito population, body water content remained similar between the two experimental conditions (ANCOVA, *ddl*=1, F=2.23, *P*=0.13). However, differences were observed among populations (ANCOVA, *ddl*=3, F=11.06, *P*<0.001) and over the duration of the experiment (ANCOVA, *ddl*=1, F=18.84, *P*<0.001). In addition, differences appeared among the populations over the duration of the experiment, as supported by the significance of interaction terms (ANCOVA, *ddl*=3, F=12.60, *P*<0.001). Although there was no variation in both RS- and ODS-reared females from Déou, and ODS-reared females from Bama, body water content progressively decreased from T0 to T7 in all other females (**Fig. 3A**).

Dry mass of mosquitoes increased from T0 to T7 in ODS-reared mosquitoes from Oursi and RS-reared ones from Bama. No significant variation of dry mass was observed in the three other colonies (**Fig. 3B**).

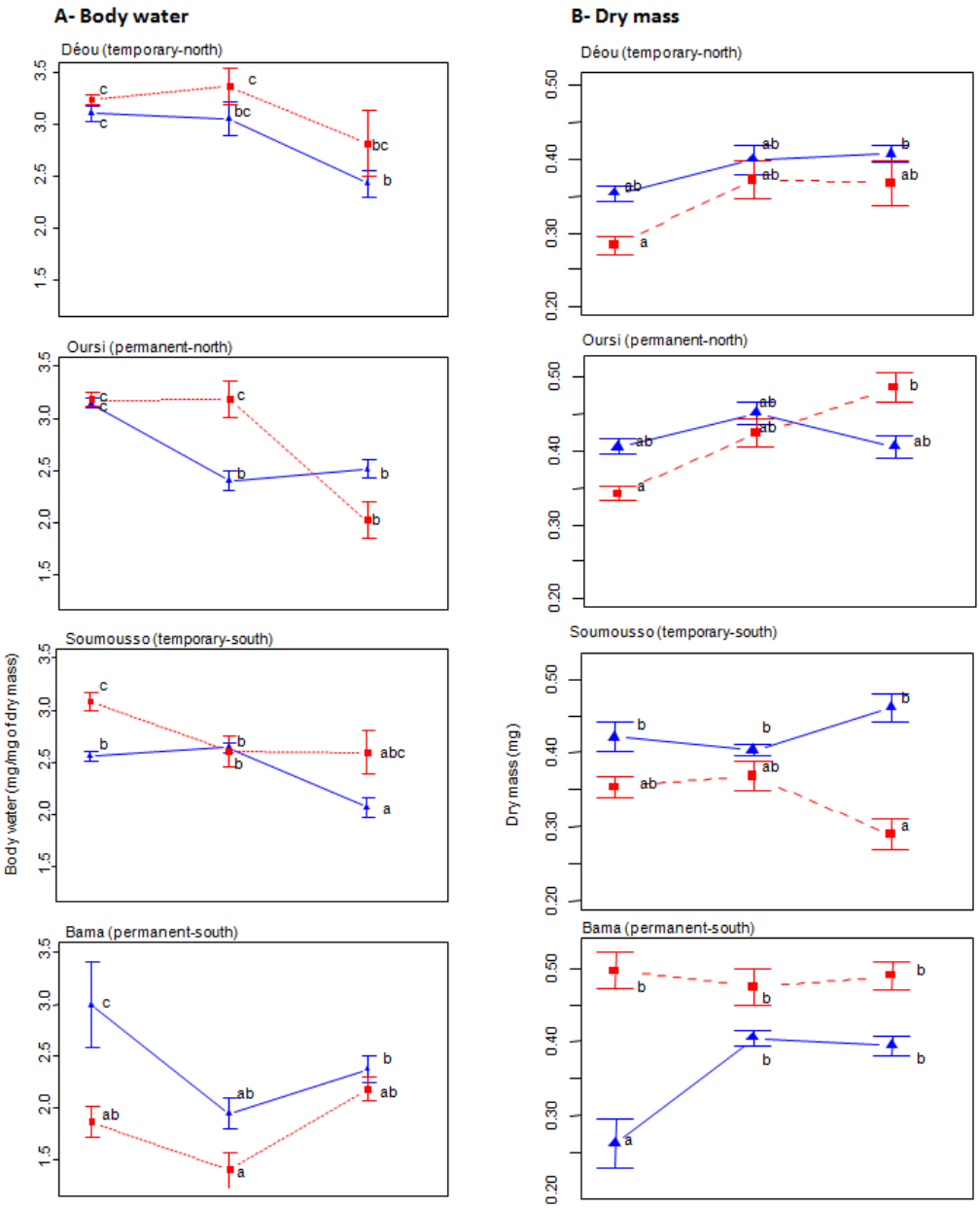


Fig. 3 Variations in body water content (A) and dry mass (B) among females *Anopheles coluzzii* from Déou, Oursi, Soumousso, Bama reared under RS (solid blue lines and triangles) or ODS (dashed red lines and squares) experimental conditions at 0, 2 and 7 days post-emergence. Values are means ± SE. Distinct letters represent significant differences among the three periods ($P < 0.05$).

3.2. Body stores

3.2.1. Protein and triglyceride contents

Protein contents were similar among the four populations reared under RS conditions (**Fig. 4A**). Protein content decreased in ODS as compared to RS conditions (ANOVA, *ddl*=3, F=7.18, *P*<0.001), and this was mostly explained by the lower protein amounts of the northernmost populations (Déou and Oursi) exposed to the ODS conditions.

Significant variations were also observed for the triglyceride contents between RS- and ODS-reared females (ANOVA, *ddl*=3, F=2.73, *P*<0.05). Larger amounts of triglycerides were also observed in Soumousso and Bama females from the ODS conditions whereas this was not the case in both northernmost populations (**Fig. 4B**). In addition, level of triglyceride contents increased from RS to ODS in females from Soumousso only.

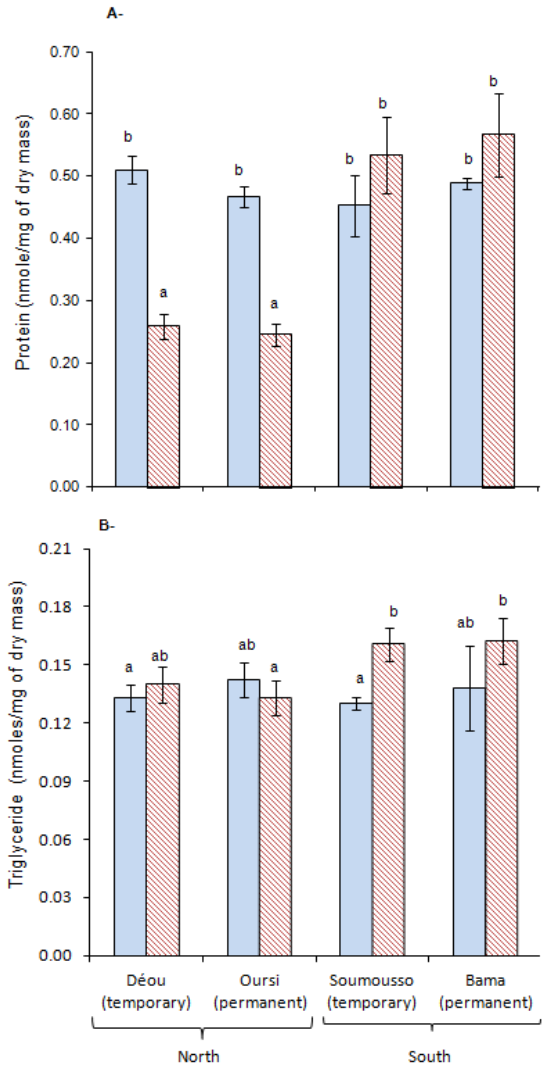


Fig.4 Stored protein **(A)** and triglycerides **(B)** contents (mmoles/mg of dry mass) ± SE in 7-days old females *Anopheles coluzzii* from Déou, Oursi, Soumousso and Bama reared under both RS (solid blue bars) or ODS (dashed red bars) experimental conditions. Distinct letters denote significant differences (*P*< 0.05).

3.2.2. Expression of Glycogen synthase, Glycogen phosphorylase and Anoga-AKHs genes

3.2.1.1. Glycogen synthase and Glycogen phosphorylase

Expression patterns of *glycogen synthase* and *glycogen phosphorylase* genes differed between the experimental conditions as a function of the population (ANOVA, *ddl*=3, F=2.90, *P*<0.05 and *ddl*=3, F=2.70, *P*<0.05, respectively). No variation between RS and ODS-reared females was observed in permanent populations (Oursi and Bama), whereas higher levels of expression of *glycogen synthase* gene were measured in RS-reared females from the temporary populations (Déou and Soumousso, 1.66 and 2.43 fold more than ODS-reared, respectively; **Fig. 5A**).

No variation of *glycogen phosphorylase* expression was observed in the northernmost populations (Déou and Oursi), whereas higher expressions of *glycogen phosphorylase* gene were measured under ODS conditions compare to RS ones in the three other populations (Soumousso and Bama: 2.16 and 1.83 fold, respectively; **Fig. 5B**).

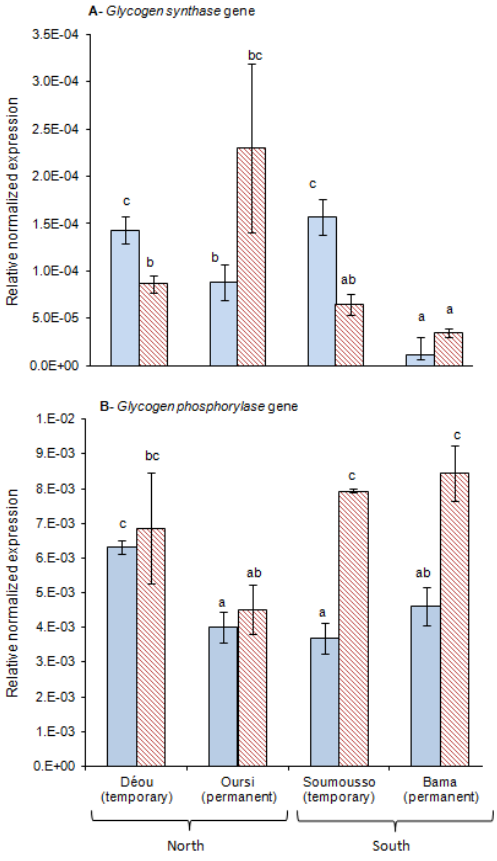


Fig. 5 Relative normalized expression of glycogen synthase **(A)** and glycogen phosphorylase **(B)** ±SE in 7-days old females *Anopheles coluzzii* from Déou, Oursi, Soumousso and Bama reared under both RS (solid blue bars) or ODS (dashed red bars) experimental conditions. Distinct letters denote significant differences (*P*< 0.05).

3.2.1.2. Anoga-AKHs

Expression patterns of *Anoga-AKHs* mRNA differed between the experimental conditions as a function of the mosquito population (ANOVA, *ddl*=3, $F_{AKH\text{-}I}$=10.62, *P*<0.001; $F_{AKH\text{-}II}$=3.68, *P*<0.05). The highest level of *Anoga-AKH-I* was measured in ODS-reared specimens from Déou (7.17 fold more than RS-reared females; **Fig. 6A**), and *Anoga-AKH-II* mRNA exhibited higher amounts in Soumousso populations maintained under ODS conditions (2.14 fold more, respectively, than RS-reared females; **Fig. 6B**).

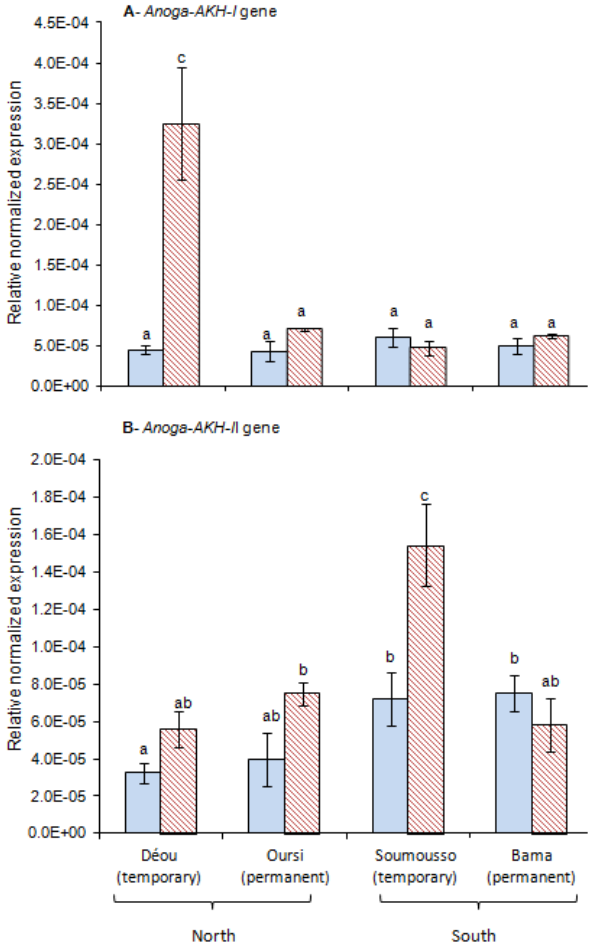


Fig. 6 Relative normalized expression of Anoga-AKH-I **(A)**, and Anoga-AKH-II **(B)** ±SE of *Anopheles coluzzii* females from Déou, Oursi, Soumousso and Bama reared under RS (solid blue bars) or ODS (dashed red bars) conditions. Distinct letters represent significant differences (*P*< 0.05).

3.3. Metabolic profiling

Among the 61 pure reference compounds, 36 were accurately detected and quantified in the samples (**Supplementary data 2**). Some of these 36 metabolites were not reliably quantified (signal/noise < 10, or metabolite concentration < quantification

limit). Therefore, three replicates were discarded from the analysis to successfully achieve the discriminant analysis (one sample from Bama and from Oursi reared under RS conditions, and one sample from Déou reared under ODS conditions).

Distinct metabolic profiles were observed among the four *An. coluzzii* populations (MANOVA, $F_{3,87}$=3.15, P<0.001), and the two experimental conditions (MANOVA, $F_{1,29}$=13.98, P<0.001). In addition, contrasted metabolic profiles were found between the experimental conditions among the different mosquito populations, as supported by the significance of interaction terms (MANOVA, $F_{3,87}$=2.44, P<0.001).

Twenty-nine out of the 36 quantified metabolites showed significant differences between at least two experimental groups, and the seven non-influential metabolites, *i.e.* glutamic acid, glyceric acid, leucine, lysine, methionine, phenylalanine, and serine were thus discarded from the subsequent LDA. The first axis (LD1) accounted for 20.08% of the total inertia, and the variation among groups was 14.65 times higher than the variation within groups (**Fig.7A**). LD1 mainly corresponded to a clear cut-off between ODS-reared females from Déou *versus* the three other populations, and RS-reared females from Soumousso *versus* the three other populations. This axis was mainly constructed by the variations of adonitol, arabitol, fructose, fructose 6 phosphate, gluconic acid, glucose 6 phosphate, glycerol phosphate, ribose, sorbitol and succinic acid (**Fig. 7A** and **C**).

The second axis (LD2) of the discriminant analysis accounted for 19.25% of the total inertia, and the variation among groups was 9.99 times higher than the variation within groups. LD2 mainly corresponded to a clear cut-off between the two experimental rearing conditions (**Fig. 7A**). Accordingly, females reared under RS conditions exhibited higher amounts of arabitol, GABA, gluconic acid, mannitol, trehalose, etc. (**Fig. 7B** and **D**). On the other hand, females reared under ODS conditions, and particularly Déou ones exhibited higher amounts of glycerol phosphate, inositol, lactic acid, maltose, proline, xylitol, etc.

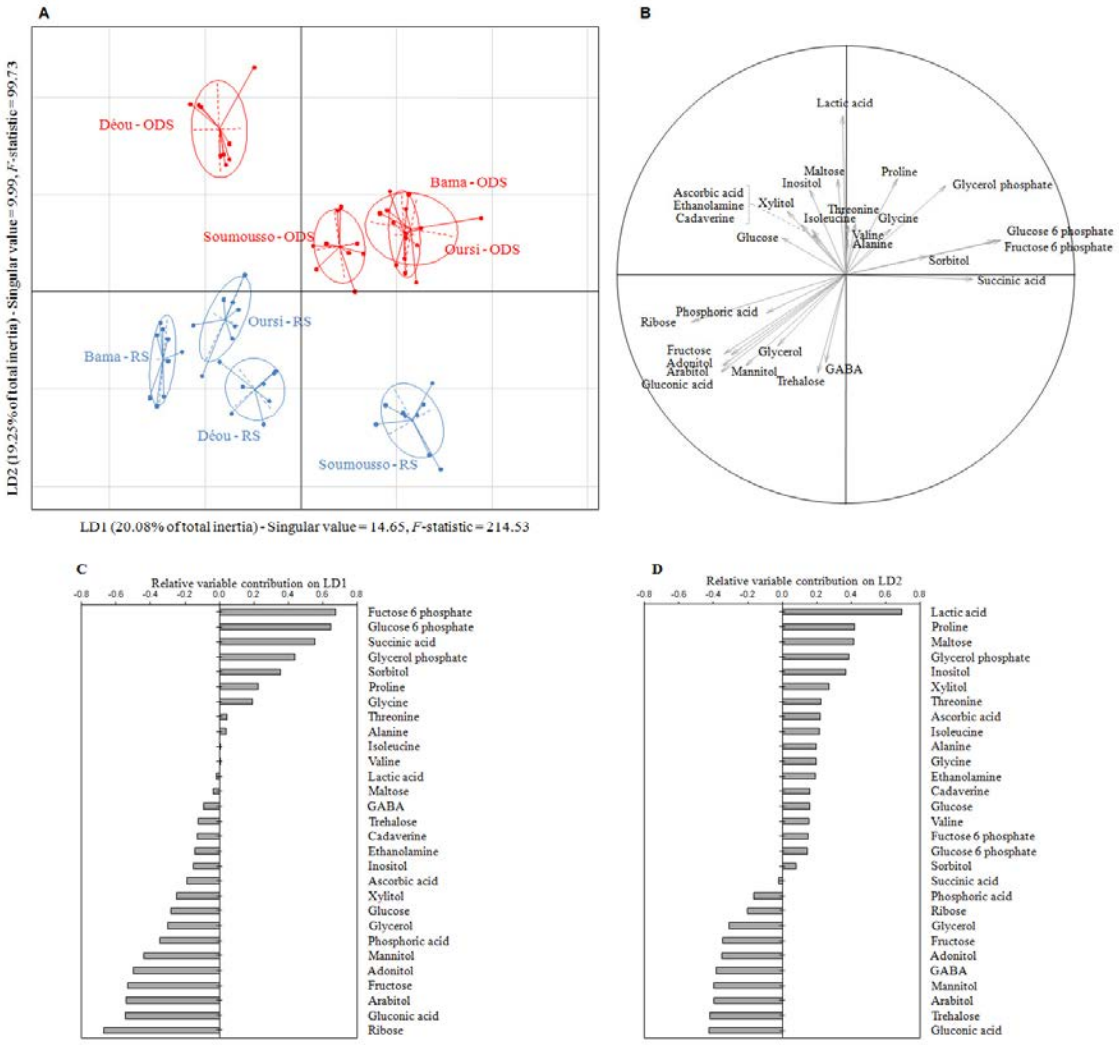


Fig. 7 (A) Sample projection of the four populations of female *Anopheles coluzzii* onto the first discriminant plane of the LDA. Blue and red samples represent specimens reared under RS and ODS conditions, respectively. The singular values correspond to the ratio between-class / within-class inertias. **(B)** The correlations circle depicts the normed relation between each metabolite and first discriminant plane. Amino acids are identified using the European abbreviation index. **(C-D)** The relative contributions of the 29 retained metabolites to LD1 and LD2.

4. Discussion

The present work compares the physiological plasticity in response to water stress in four distinct populations of female *An. coluzzii* sampled from different habitats in Burkina-Faso. Biochemical and physiological differences were observed when these mosquitoes were reared under contrasted environmental conditions (ODS, RS). In particular, exposure to dry conditions (ODS) elicited (i) distinct metabolic

rearrangements including decreased amount of protein reserves, (ii) increased levels in the expression of *glycogen phosphorylase* genes suggesting the use of glycogen stores, accumulation of osmoprotectants (*i.e.* glycine, inositol and sorbitol) and (iii) intermediately metabolites from the pentose phosphate. These rearrangements differed among anopheline populations, suggesting physiological response to water stress depend on the geographical origin of mosquitoes and thus of local selective pressure.

Previous investigations performed on female *An. coluzzii* pointed out the enhanced desiccation resistance of these malarial mosquitoes at the onset of the dry season (Article I; Lee *et al.*, 2009; Mamai *et al.*, 2014). In the present study, the body water loss of RS- and ODS-reared females maintained in their respective growing conditions remained similar over time in all populations. These results suggest that mechanisms preventing body water loss are elicited in ODS-reared mosquitoes. Preventing body water loss requires, among other changes, physiological adjustments in the nature of the body reserves that are catabolised (Chown *et al.*, 2010; Schmidt-Nielsen, 1990). Glycogen degradation is the major source of energy used by water-stressed insects (Gibbs, 2002; Gibbs *et al.*, 1997; Schmidt-Nielsen, 1990). However, our data suggest the use of stored proteins and lipids too. Indeed, results showed a decreased level of stored proteins at ODS in the two northernmost populations of Déou and Oursi, and conversely an increased level of stored triglycerides in the ODS-reared temporary South population of Soumousso. We hypothesise that these changes may help mosquitoes from Déou, Oursi and Soumousso in supplying the energetic demand needed to prevent body water loss during the dry season. For instance, we presumed that the release of amino acids induce by the degradation of stored proteins in the two northernmost populations (Déou and Oursi) may be used to fuel the synthesis of metabolites with osmoprotective roles at ODS (Patrick & Bradley, 2000; Rathinasabapathi, 2000). In the present work, we did not measure the variations of glycogen, but molecular data from the level expressions of *glycogen phosphorylase* and *glycogen synthase* genes demonstrate an up-regulation of the gluconeogenesis at ODS in seven-day old females from the two southernmost populations of Soumousso and Bama. These results suggest that the energetic reserves that must

be catabolised to prevent body water loss in female mosquitoes may highly depend of their geographical origin. In addition, except in permanent populations from Oursi and Bama, mRNA expression of *glycogen synthase* was slightly decreased or remained constant in the two temporary populations (Déou and Soumousso). This finding may result from the *ad libitum* availability of 10% glucose solution in all experimental conditions, triggering a similar mRNA expression for this enzyme between the two conditions in seven-day old females.

Data from the metabolic profiles supported the hypothesis of an up-regulation of the gluconeogenesis and glycolysis activities in the ODS-reared mosquitoes, as shown by increased levels of free-circulating glucose in all populations. However, it is uncertain if this glucose is used in ODS-reared anopheline as a substrate for the synthesis of other metabolites (Storey & Storey, 1985; Teets *et al.*, 2013; Ziegler *et al.*, 1979), or if it has osmoprotective role (Elnitsky *et al.*, 2008). Our analysis showed that both glucose and fructose 6 phosphate were significantly accumulated in females from Bama, Oursi and Soumousso when they were exposed to ODS conditions. These two sugars can be initial and end products, respectively, of the pentose phosphate pathway which is often elicited in insects exposed to desiccation conditions (Michaud & Denlinger, 2004; Timmermans *et al.*, 2009). Indeed, pentose phosphate pathway is the source of major reductants (*i.e.* NADPH) and osmoprotectant precursors (Koštál *et al.*, 2004; Kruger & von Schaewen, 2003; Storey & Storey, 1991). Accordingly, elevated amounts in sorbitol, inositol and glycerol phosphate, which have similar protective roles as the heat shock proteins (Yancey, 2005) were also measured in ODS-reared mosquitoes. Increased levels of amino acids, like the glycine and proline were also measured in ODS-reared females. These two amino acids have often been observed in arthropod species in order to counterbalance the deleterious effects of dehydration (Article V, VII; Yancey, 2005).

In any given species, populations from distinct habitats are exposed to different environmental selective pressures resulting in the expression of distinct phenotypes and local adaptation. Recent morphological and physiological investigations suggest that populations of *An. coluzzii* express a wide range of

phenotypes according their geographical origin and the environmental conditions they experienced during their development (Article IV; Yaro *et al.*, 2012). In particular, there are expectations that phenotype heterogeneity in these species match with distinct aestivation abilities of local mosquito populations (Yaro *et al.*, 2012). Accordingly, mosquitoes can be "strong aestivators" programmed to engage into a dormant state at ODS, or "weak aestivators" of which induction of dormant state depend on the availability of surface water in the habitat to finish the gonotrophic cycle and lay eggs (Yaro *et al.*, 2012). In the present work, this assumption was supported, as the water availability (temporary, permanent) seems to exert a stronger influence on the metabolic profiles of ODS-reared mosquitoes. This was particularly manifest for females from permanent water availability (Bama and Oursi), whose metabotypes highly overlapped. The metabolic differentiation was slightly different in females from temporary water availability (Déou and Soumousso), suggesting that their geographical origins (North, South) influence their physiological adjustments too. Of note, the temporary population of Déou exhibited very distinct metabolic profiles as compared to the three other populations. These females were characterised by an important level of lactic acid and sugars like the maltose, inositol and glucose compared to the three other populations exposed to the ODS conditions too. In particular, accumulation of lactic acid suggests that these females face severe water and/or hypoxia stress at ODS, and they thus elicits anaerobic metabolism (Hoback & Stanley, 2001; Michaud & Denlinger, 2007; Schilman *et al.*, 2011; Verberk *et al.*, 2013). Anaerobic metabolism is well known to replace the aerobic one during dormancy in insects (Hahn & Denlinger, 2011), as it minimise the energetic expenditure and water loss by gas exchange activity. Increase of this acid may suggest a "strong aestivator" phenotype in these females at the onset of the dry season.

The molecular analysis of the two *Anoga-AKH-I* and *-II* genes supports rearrangements in energetic metabolism of the mosquitoes at ODS, and these changes depend on the water availability in their habitat. Indeed, both AKH peptides were over-expressed at the onset of the dry season in females sampled in habitats with temporary water ponds (*i.e.* Déou and Soumousso). Moreover, the nature of AKH

peptide over-expressed changed according they issued from the North or the South of the Burkina-Faso.In particular, the *Anoga-AKH-I* gene was specifically over-expressed in ODS-reared females from Déou, whereas the *Anoga-AKH-II* gene was over-expressed in ODS-reared females from Soumousso. AKH peptides are known to mobilise and transport energetic reserves from fat body to hemolymph. Moreover, they enhance the gluconeogenesis by up-regulating the glycogen phosphorylase activity and prompt the release and transport of osmoprotectants. Recent expectations suggest that increased level of AKH peptide are involved in the adjustments of energetic and nutrient homeostasis in insects entering in overwinter diapause (Hahn & Denlinger, 2011; Isabel *et al.*, 2005; Kodrík *et al.*, 2003; Lee & Park, 2004; Socha & Kodrík, 1999; Woodring *et al.*, 2002). Assuming that females *An. coluzzii* from habitats, where water ponds disappear at the onset of the dry season, are strong aestivators (Yaro *et al.*, 2012), we hypothesise the increase level of both *Anoga-AKH-I* and *–II* genes in females issued from these habitats (Déou and Soumousso) constitute markers of a strong aestivation strategy in mosquitoes. However, interpret why mosquitoes over-expressed the *Anoga-AKH-I* than the *Anoga-AKH-II* genes, and vice versa, is obviously tentative because the exact role of each peptide still unknown (Kaufmann & Brown, 2008, 2006). Notwithstanding, results showed that the level of variations of the *Anoga-AKH-II* gene match with those of the *glycogen phosphorylase* one, suggesting a regulating role of this AKH peptide on the use of stored glycogens in the mosquitoes.

5. Conclusion

Overall, we found that female *An. coluzzii* exhibited distinct physiological and biochemical rearrangements in response to water stress at ODS, and these changes depend on the geographical origin of mosquitoes, suggesting adaptation/acclimation of populations to local environmental conditions. As we expected, we found that water availability in the habitat (*i.e.* permanent *versus* temporary population) exerts a strong influence on the metabolic profiles and the molecular expression levels of the AKH peptides in female mosquitoes. However, further studies are needed to explore if such differences may conduct to distinct

aestivation abilities in these malarial mosquitoes. Of particular interest, we found that the metabolic rearrangements also depend of the latitudinal origin of mosquitoes, as showed, for instance, by the distinct metabolic profile of females from Déou. The nature of body reserve that must be catabolised at ODS also differed according North and South populations. Although the first one preferentially catabolised the stored proteins, the second catabolised the stored glycogens. Additional studies are needed to explore the ecophysiological and biological differences between North and South populations of *An. coluzzii* in the Burkina-Faso, as it seems it exerts significant influence on the physiological and biochemical responses of females to water stress at ODS.

Acknowledgements

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Supplementary data

Supplementary data 1. Nucleotide sequences of primers used in polymerase chain reaction and 5'/3' RACE for the amplification of Rps13 (reference gene), *Anoga-AKHs*, *Glycogen synthase* and *Glycogen phosphorylase* (target genes) sequences in *An. gambiae*.

Primer	Direction	Sequences (5'-3')
Rps13	FOR	TATTTCCAAATCCGCGCTAC
Rps13	REV	CATGATACGCAGCACCTTGT
Anoga-AKH-I	FOR	TGCTGATTTGTGCCTCTTTG
Anoga-AKH-I	REV	ATTCCCCAACCCTACCTGAA
Anoga-AKH-II	FOR	CGCTGGACAGGTAACGTTTT
Anoga-AKH-II	REV	GACTCATCCGTTTGCAGTGA
Glycogen synthase	FOR	GGGCTGAACGTGAAGAAGTT
Glycogen synthase	REV	GCCAATGCCTCGATAAAGAT
Glycogen phosphorylase	FOR	CGGCCTCGATGTTTGATATT
Glycogen phosphorylase	REV	ACCGCACAGATGAGCTTGAT

Supplementary data 2. List of the 36 metabolites detected in females of *Anopheles coluzzii*.

Compound abbreviations in brackets
Free amino acids
Alanine (Ala)
Glutamic acid (Glu)
Glycine (Gly)
Isoleucine (Ile)
Leucine (Leu)
Lysine (Lys)
Methionine (Met)
Phenylalanine (Phe)
Proline (Pro)
Serine (Ser)
Threonine (Thr)
Valine (Val)
Sugars
Fructose
Fructose-6-phosphate
Glucose
Glucose-6-phosphate
Maltose
Ribose
Trehalose
Polyols
Adonitol
Arabitol
Glycerol
Glycerol-3-phosphate
Inositol
Mannitol
Sorbitol
Xylitol
Intermediate metabolites
Succinic acid
Other metabolites
Ethanolamine
Ascorbic acid
Cadaverine
Gamma-aminobutyric acid (GABA)
Gluconic acid
Glyceric acid
Lactic acid
Phosphoric acid

2. Temps de développement pré-imaginal et plasticité morphologique d'*An. coluzzii* (Article IV)

Selon les travaux précédents, il existe une variation de la plasticité physiologique des femelles *An. coluzzii* en réponse aux conditions environnementales de la saison sèche selon leur l'origine géographique (Article II, III). Ces résultats suggèrent notamment que les populations de moustiques subissent des pressions locales conduisant à des mécanismes d'adaptation/acclimatation différents entre les populations. Si ces réarrangements physiologiques ont une réelle valeur adaptative, ils doivent alors être liés à des changements phénotypiques mesurables à d'autres échelles. Ces changements se traduiraient notamment par des différences dans la durée du développement pré-imaginal (un indicateur connu du niveau d'adaptation locale des populations d'insectes ; Czarnoleski *et al.*, 2013; Kingsolver *et al.*, 2009) et dans la morphologie des adultes émergeants (une conséquence directe de la qualité de l'environnement perçue pendant la vie larvaire chez de nombreux insectes, y compris *An. gambiae* ; Aboagye-Antwi & Tripet, 2010 ; Lee *et al.*, 2013). Il est en effet admis que les variations des paramètres environnementaux pilotent le taux et la durée de développement des insectes (Couret *et al.*, 2014; Damos & Savopoulou-Soultani, 2012; Lyons *et al.*, 2013; Mouline *et al.*, 2012), et altèrent leurs propriétés morphométriques telles que la forme et la taille (Atkinson, 1994; Czarnoleski *et al.*, 2013; Kingsolver *et al.*, 2009; Pétavy *et al.*, 2004).

Parmi ces changements morphométriques, les variations de la forme et de la taille des ailes des moustiques renferment de nombreuses informations concernant les événements démographiques et génétiques des populations (Dujardin, 2008, 2011). En effet, les paramètres géo-morphométriques des ailes de moustiques dépendent de processus de sélection locale, et notamment des conditions environnementales perçues pendant le développement larvaire (Andersen *et al.*, 2005; Ayala *et al.*, 2012 ; Morales Vargas *et al.*, 2013; Soto *et al.*, 2006). Les variations de ces paramètres ont ainsi été le sujet de nombreuses études chez les insectes afin de caractériser les populations et leur adaptation aux conditions environnementales de leur habitat (Haas and Tolley, 1998; Hoffman and Shirrifs, 2002; Morales Vargas *et al.*, 2013; Roggero and d'Entrèves, 2005).

Dans le cadre de ce travail, nous avons examiné la plasticité développementale et morphologique de cinq populations d'*An. coluzzii* issues de cinq localisations géographiques différentes. Pour cela, le temps de développement des stades pré-imaginaux et la plasticité géo-morphométrique des ailes des femelles *An. coluzzii* ont été évalués après élevage en conditions de saison des pluies (RS) et du début de la saison sèche (ODS). Le temps de développement larvaire (de l'éclosion à l'émergence) a été mesuré, et la masse sèche corporelle des femelles à l'émergence a été déterminée pour chaque population dans les deux conditions d'élevage. La taille, la surface et la forme de l'aile droite des femelles ont ensuite été déterminées par des méthodes de géométrie morphométrie modernes. Ces paramètres morphométriques ont été analysés en fonction des conditions environnementales et de l'origine géographique des femelles *An. coluzzii*.

Seasonal variation in wing size and shape between geographic populations of the malaria vector, *Anopheles coluzzii* in Burkina-Faso (West Africa) - (Article IV)

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Abstract

The mosquito, *Anopheles coluzzii* is a major vector of human malaria in Africa with widespread distribution throughout the continent. The species hence populates a wide range of environments in contrasted ecological settings often exposed to strong seasonal fluctuations. In the dry savannahs of West Africa, this mosquito population dynamics closely follows the pace of surface water availability= the species pullulates during the rainy season and is able to reproduce throughout the dry season in areas where permanent water bodies are available for breeding. The impact of such environmental fluctuation on mosquito development and the phenotypic quality of emerging adults has however not been addressed in details. Here, we examined and compared phenotypic changes in the duration of pre-imaginal development, body dry mass at emergence and wing size, shape and surface area in young adult females *An. coluzzii* originated from five distinct geographic locations when they are reared in two contrasting conditions mimicking those experienced by mosquitoes during the rainy season (RS) and at the onset of the dry season (ODS) in Burkina-Faso (West Africa). Our results demonstrated strong phenotypic plasticity in all traits, with differences in the magnitude and direction of changes between RS and ODS depending upon the geographic origin, hence the genetic background of the mosquito populations. Highest heterogeneity within population was observed in Bama, where large irrigation schemes allow year-round mosquito breeding. Further studies are needed to explore the adaptive value of such phenotypic plasticity and its relevance for local adaptation in *An. coluzzii*.

Key words: Mosquito; development; phenotypic plasticity; dry season; geometric morphometrics.

List of abbreviations: RH = Relative Humidity; RS = Rainy Season; ODS = Onset of the Dry Season; LM = landmarks ; CS = Centroid Size; GPA = Generalized Procrustes Analysis; PCA = Principal Component Analysis ; PC1 = First axis of the Principal Component Analysis; PC2 = Second axis of the Principal Component Analysis; ΔD = vector length; θ = angular difference; MD = Metric Disparity

1. INTRODUCTION

Species living in dry savannahs of West Africa, including the malarial mosquito *Anopheles coluzzii* (Diptera, Culicidae), have to face high seasonality in their environment, with the occurrence of an adverse dry season during which mean temperatures rise, relative humidity decreases, and water collections dry up. In such environment, the population dynamics of anopheline species follow the pace of water collections availability (Adamou *et al.*, 2011; Lehmann *et al.*, 2014, 2010; Yaro *et al.*, 2012). However, larval instars of *An. coluzzii* can be found in both temporary (*i.e.* rain-filled) and permanent (*i.e.* generally man-made) water collections (Costantini *et al.*, 2009; Gimonneau *et al.*, 2012). Hence, in areas where large anthropogenic (*e.g.* dams, ricefields, etc.) or natural (*e.g.* ponds, river edges, etc.) surface water collections are available all year round, mosquitoes persist and reproduce all year long, whereas in areas where only temporary waters are available, local mosquito populations virtually disappear during the dry season. In such area, *An. coluzzii* females might enter a state of quiescence/diapause and persist throughout the dry season by aestivation (Lehmann *et al.*, 2010, 2014). In this context, mosquitoes may have developed local behavioural, physiological and/or morphological adaptations to survive and persist during the adverse conditions of the dry season. There is indeed evidence in the recent literature for seasonal physiological adjustments in some anopheline species, including *An. coluzzii* (Article I; Adamou *et al.*, 2011; Huestis *et al.*, 2012, 2011; Huestis & Lehmann, 2014; Lehmann *et al.*, 2010; Mamai *et al.*, 2014; Yaro *et al.*, 2012). Whether these physiological changes sustain variation in other fitness traits of the mosquito remains unknown and their evolutionary and adaptive value has yet to be assessed.

Environmental conditions (*i.e.* biotic and abiotic factors) perceived by larvae during development are known to pilot insect's developmental rate (Couret *et al.*, 2014; Damos & Savopoulou-Soultani, 2012; Lyons *et al.*, 2013; Mouline *et al.*, 2012) with consequences on adults' overall phenotypic quality, including their morphometric properties (*i.e.* shape, size, asymmetry) (Aboagye-Antwi & Tripet, 2010; Atkinson, 1994; Czarnoleski *et al.*, 2013; Kingsolver *et al.*, 2009; Pétavy *et al.*, 2004). Morphological approaches have recently received increasing attention mainly with the

advent of new applications and current developments in geometric morphometrics (Ayala *et al.*, 2011; Sadeghi *et al.*, 2009; Zimmermann *et al.*, 2012). Although morphometric methods were traditionally used at the upper taxonomic level (*i.e.* at the genus/species level), geometric morphometrics now offers powerful analytical and graphical tools for quantitative assessment and visualisation of morphological variations within and among species. Currently, geometric morphometric approaches are increasingly applied to a wide range of research fields including systematics, phylogeny and population genetics, ontogeny and developmental stability (Debat *et al.*, 2011; Klingenberg & Marugán-Lobó, 2013; Klingenberg & McIntyre, 1998; Morales Vargas *et al.*, 2013; Savriama *et al.*, 2012). One of the main added values of geometric morphometrics is its ability to consider the variation of both size and shape of individuals and/or organs separately. As such, wings have been the subject of many geometric morphometric analyses in insects (Baylac & Daufresne, 1996; Rohlf & Slice, 1990), and many of these studies characterised populations within species among geographic and climatic variations (Haas & Tolley, 1998; Hoffman & Shirrifs, 2002; Morales Vargas *et al.*, 2013; Roggero & d'Entrèves, 2005). Indeed, it was shown that wing shape variation can inform on current or recent population events and contains a great deal of information on genetic variation among populations (Dujardin, 2011, 2008). On the other hand, insect wing size, often used as a proxy of whole-insect size, has been shown to vary chiefly according to larval growth conditions (Koella & Lyimo, 1996; Lyimo *et al.*, 1992; Mouline *et al.*, 2012). Recently, Andersen *et al.* (2005) showed that both size and shape of the wing of *Drosophila mercatorum* (Diptera, Drosophilidae) changed as a response to the maternal and developmental temperature. Similarly, Ayala *et al.* (2011) further argued that changes observed in wing morphometric properties of *Anopheles funestus* mosquitoes are the result of natural selection and may contribute to local adaptation in wild populations of this mosquito. Accordingly, developmental plasticity expressed under various environmental conditions may result in morphological changes in adults that contribute to local adaptation.

To test this hypothesis, we monitored larval development and assessed adult phenotypic variation in five ecologically and geographically distinct populations of

An. coluzzii exposed to contrasted environmental cues mimicking the rainy and dry season conditions in Northern Burkina-Faso. Desiccation has been proposed as a major threat when larvae develop in temporary waters. It is therefore expected that mosquito populations adapted to breed in rain-dependant collections will speed-up their development in response to increased desiccation threat in dry season conditions. On the other hand, because drying-out of the breeding site is not a threat when larvae develop in permanent waters, we predicted that the duration of larval development will increase in the dry season conditions, reflecting suboptimal growth conditions (*e.g.* exposure to extreme temperatures and fluctuations thereof). The impact of changing developmental duration on the overall adult fitness should vary according to the level of local adaptation of the population under scrutiny. Here, we used wing geometric morphometrics and we monitored dry weight at emergence to explore phenotypic plasticity in emerging adult females when larvae were reared under contrasted environmental conditions.

2. MATERIALS AND METHODS

2.1. Sample populations

In Burkina-Faso (West Africa), annual rainfalls and duration of the rainy and dry seasons vary along a latitudinal gradient. Although, rainfalls do not exceed 300 mm during the 2-3 months of the rainy season in the northernmost regions (*i.e.* 9-10 months of dry season), it can be as high as 1,200 mm during the 5-6 months of the rainy season in the southernmost regions (*i.e.* 6-7 months of dry season; see **Fig. 1**). The experiments were conducted using four mosquito populations derived from the progeny of wild-caught *An. coluzzii* females from North and South Western Burkina-Faso. Gravid female mosquitoes were sampled from within human dwellings in localities where *An. coluzzii* mosquitoes are present all year long (*i.e.* presence of year-round mosquito breeding opportunities in Oursi [14°40'N, 0°27'W] and Bama [12°01'N, 04°23'W]) and in localities where no permanent breeding is possible and the mosquito populations are highly seasonal (*i.e.* Déou [14°35'N, 0°43'W] and Soumousso [11°01'N, 04°02'W]; Figure 1). Before experiments, all populations were reared for 6 generations at the Institut de Recherche en Sciences de la Santé (IRSS) in

Bobo-Dioulasso under controlled conditions (27 ± 1 ºC, 80 ± 10% relative humidity with LD cycles of 12h=12h). Females were routinely blood fed on restrained rabbits, their eggs were collected on filter paper and larvae were reared in trays and fed grounded fish food (Tetramin®) *ad libitum*. For experiments, we included a fifth sample consisting of *An. coluzzii* mosquitoes from the Ngousso colony originated from equatorial South Cameroon [3°52'N, 11°31'E] in 2006. Mosquitoes from this last colony were further considered as a control colony because they were maintained in laboratory conditions for >50 generations (Harris *et al.*, 2010) and thus were not adapted to fluctuating environmental conditions.

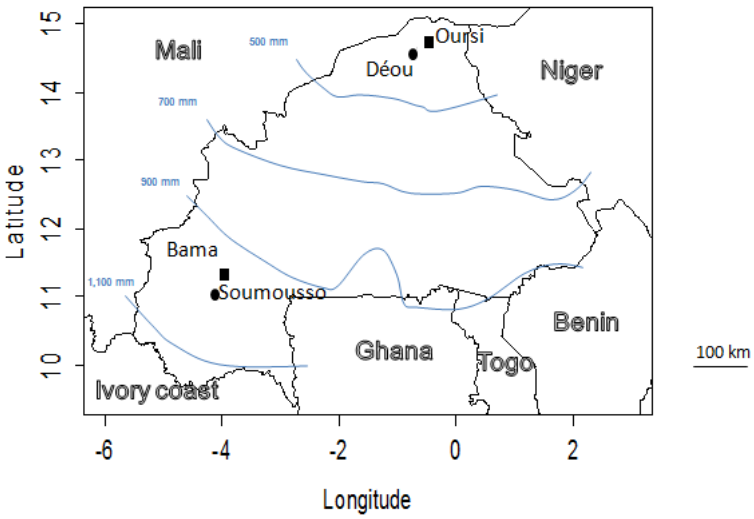

Fig. 1 Geographical localisation of the four localities where wild *An. coluzzii* mosquitoes were collected in northern and south-western regions of Burkina-Faso (West Africa). Squares represent permanent mosquito populations and circles represent temporary populations (see text). Blue lines represent mean annual rainfall in mm (derived from Clavel *et al.*, 2009).

2.2. Experimental rearing conditions

Mosquitoes of the five populations were reared from eggs to adults in programmable climatic chambers (Sanyo MLR 315H, Sanyo Electric Co., Osaka, Japan) parameterised to reproduce ambient daily climatic variation (*i.e.* temperature, relative humidity [RH] and photoperiod duration) experienced by mosquitoes in Oursi, the northernmost region of Burkina-Faso, during the rainy season and at the onset of the dry season. Hourly temperature and RH, and photoperiod duration were set up using the climatic data available at http=//www.gaisma.com/ for the Oursi area, averaged over the past 30 years for a period ranging from August 1st to 31th (*i.e.* rainy season, hereafter referred to as RS conditions) and from December 1st to 31th (*i.e.* at the onset of the dry season, hereafter referred to as ODS conditions). Climatic conditions in the incubators (N=1 for RS conditions, N=2 for ODS conditions) mimicked those

observed in the fields and were tightly monitored using MicroLog Pro monitors placed inside the incubators and inside one of the rearing pans to monitor larval rearing water temperature (EC750, Davis Instruments, Hayward, CA, USA; see **Fig. 2**).

For each population, 3-4 independent sessions from fresh batches of eggs obtained from more than 50 caged gravid females were used to rear mosquitoes. For each session, two batches of eggs were merged in order to achieve large sample sizes and synchronous hatching. For each climatic chamber (N=3), the experimental conditions (RS, ODS) were switched between the sessions.

Upon collection in the population cages, eggs were transferred into independent plastic trays (30 cm × 20.5 cm × 6.5 cm) containing 1 L of deionised water, and immediately exposed to the RS or ODS experimental conditions in the climatic chambers. After hatching, first larval instars were readily transferred into new plastic trays filled with 1 L of deionised water at an optimal growing density of 200 larvae per tray to reduce variation in adult body size at emergence. Four to six plastic trays (*i.e.* 800-1,200 larvae) were used for each mosquito population and each experimental condition (RS, ODS). Every day, the position of the trays was randomly alternated to avoid positional effects within the incubators. Larvae were fed daily *ad libitum* with sprinkled ground fish food (Tetramin®) until pupation. Pupae were collected and immediately transferred into plastic cups (diameter 7 cm × height 8.5 cm) filled with 10 mL of deionized water, and maintained under RS or ODS conditions until adult emergence. Males were discarded and the right wing of adult females was dissected out and mounted onto microscope slides for further geometric morphometrics analyses.

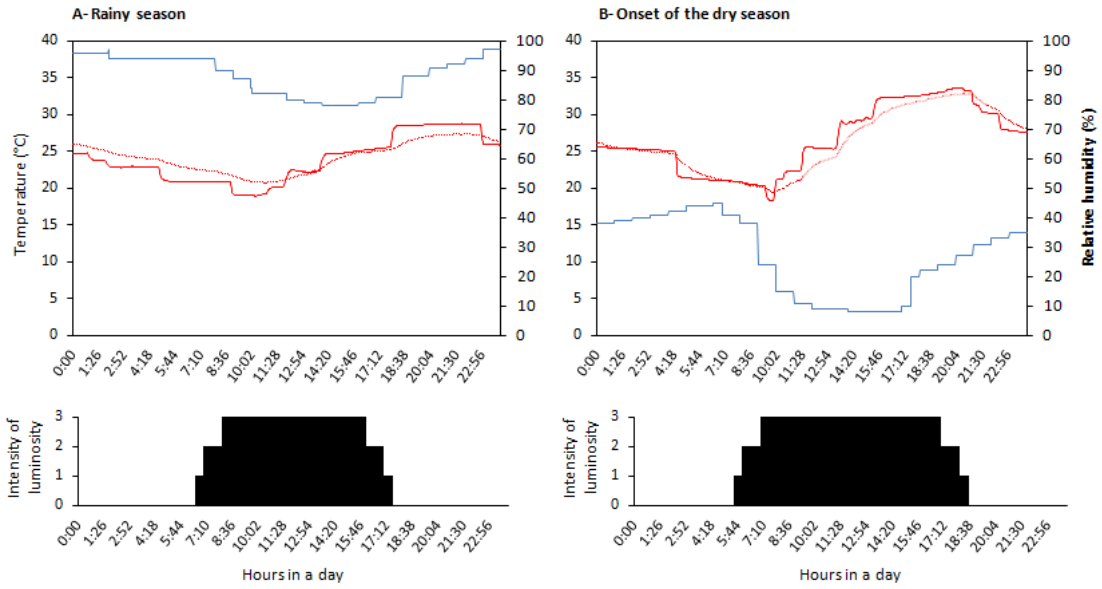

Fig. 2 Daily fluctuations in air (solid red lines) and rearing water (dashed red lines) temperature (°C) and relative humidity (% – solid blue lines) used in the climatic chambers to mimic environmental variations in Oursi (A) during the rainy season (RS), and (B) at the onset of the dry season (ODS). Photoperiod duration is represented by the black bars below.

2.3. Pre-imaginal development and body dry mass

Duration of the pre-imaginal development was monitored in each mosquito population reared in both RS and ODS conditions. Every 12 hours, live larvae and pupae were counted and transferred into new plastic trays to prevent scum formation and accumulation of toxic waste metabolites. Duration of the pre-imaginal development was measured from egg hatching to adult emergence.

For each anopheline population and each treatment (RS, ODS), a subset of freshly emerged (*i.e.* <1-h old) individuals issued from the different rearing sessions were randomly sampled and dried for 3 days at 60 °C. Dried females were then weighted using a microbalance (Sartorius SE2, d=1µg) to obtain the mosquito's dry mass.

2.4. Wing geometric morphometry

2.4.1. Sample preparation and data acquisition

Pictures of the detached right wing of female mosquitoes were captured using a Religa 2000R Qimaging Iast 1394 digital camera connected to a binocular microscope (x40). Pictures were calibrated, and the wing was put at the center of the visual field to allow

accurate size comparisons, and to reduce the risk of optical distortion. As recommended by several authors a set of 12 landmarks (LM) per wing was digitised (Jirakanjanakit *et al.*, 2008, 2007; Morales Vargas, 2013; **Fig. 3**). Damaged wings were not used to perform this assay.

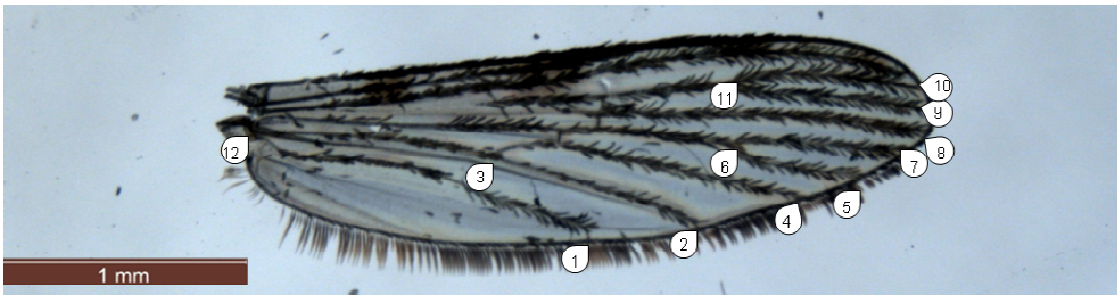

Fig. 3 Position of the different landmarks (LM) digitised on the dorsal face of the right wing of females *Anopheles coluzzii*. LM1 to 11 are type I landmarks, whereas LM12 is type II landmark defined as the transition between the alula and the posterior margin of the wing.

2.4.2. Wing size, shape and surface area

Wing size variation between experimental conditions (RS, ODS) and among the five populations of *An. coluzzii* was investigated using the isometric estimator Centroid Size (CS) derived from coordinate's data (Jirakanjanakit *et al.*, 2008, 2007; Jirakanjanakit & Dujardin, 2005). CS is defined according the following equation= CS = $\sqrt{(\sum d^2)}$, where d represents the distance between the configuration centre of LM and each individual LM (Bookstein, 1997).

Wing shape conformations were obtained using the Generalized Procrustes Analysis (GPA; Rohlf & Slice, 1990) superimposition algorithm and the subsequent projection of the Procrustes residuals into Euclidian space. Both non-uniform (local variation) and uniform (global variation such as stretching and compression) components were examined to describe the differences in shape as deviation from an average configuration of LMs. The Collyer & Adams (2007) procedure was used to measure the wing shape vector phenotype changes occurring between the two experimental rearing conditions among the five populations, and defined in terms of the magnitude of change (vector length; ΔD) and the direction of change (angular difference; θ).

Wing surface area of mosquitoes was measured using the area of the digitised polygon.

2.5. Statistical analyses and software used

2.5.1. Statistical analyses

Before analysis, normality distribution and homoscedasticity of the pre-imaginal development duration and dry mass datasets were verified using Shapiro-Wilk and Bartlett's tests, respectively. We first tested the effect of the rearing session on both pre-imaginal development duration and body dry mass of female mosquitoes. Then, because no significant effect was found we used two-way ANOVAs to compare pre-imaginal development duration and body dry mass between the two experimental rearing conditions (RS, ODS) and among the five mosquito populations. Tukey's *post-hoc* procedures were then used to perform comparisons among the levels of significant factors.

The relative geometric morphometric measurement error was first measured in both size and shape variables (see **Supplementary data 1**). Then, Procruste ANOVAs were used to test the effect of the rearing session on both size and shape variations in female *An. coluzzii*. Because, we did not find a significant effect, wing size variation between experimental conditions and among populations was then investigated using an ANOVA procedure followed with Tukey's *post-hoc* when required. Wing shape variables were analysed using a MANCOVA analysis with experimental conditions (RS, ODS), mosquito populations and CS as explicative factors. Because the scaling for centroid size does not remove the allometric changes of size and their influence on shape, a multivariate regression test was computed with size as independent variable and shape as dependent one. Statistical significance of the multivariate regressions was assessed using non-parametric permutation tests (10,000 permutations). A principal component analysis (PCA) was then used to graphically represent wing shape conformation between RS and ODS conditions, and among the five populations. The corresponding mean shape change along the two first component axes was examined showing configuration changes at a magnitude scale of 3.0. Statistical significance of the PCA was checked by permutation tests (10,000 permutations). A neighbour joining tree on the Mahalanobsis distances derived from the conformation variables was also examined. The significance of ΔD (vector length) and θ (angular difference) calculated using the procedure described in Collyer & Adams (2007) was tested using 5,000 random permutations.

Wing surface area of mosquitoes was measured using a TclTk script (derived from the R. Suchenwirth's algorithm available at http://wiki.tcl.tk/12081) computing the area of the digitised polygon considered as an assemblage of triangles, each one defined by their corner coordinates (use of Heron's formula). Because there is expectation that CS and surface area are redundant information, we then tested such correlation using a Pearson test and a linear regression model.

An analysis of the metric disparity of the wing conformation was performed and compared between populations and among experimental rearing conditions using Student's t tests.

2.5.2. Software used

The CLIC90 software developed by Dujardin & Slice (2007) and Dujardin (2008) was used (i) to perform the LM digitisation, (ii) calculate the superimposition coordinates, the wing CS and surface area, and (iii) perform the Collyer & Adams (2007) procedure. CLIC90 was also used to build the neighbour joining tree based on the Mahalanobis distances and the analysis of the metric disparity, both derived from the conformation variables.

We used the R 2.15.0 software (R Development Core Team, 2008) to perform the different permutation tests, Tukey *post-hoc* tests, PCA and statistical variance analyses.

3. RESULTS

3.1. Pre-imaginal development and body dry mass

A total of 4005 female mosquitoes (266 to 626 individuals for each population and treatment) were used to conduct the assay. Analyses showed that pre-imaginal development duration significantly changed between the rearing conditions and that these changes differed among the mosquito populations considered, as shown by the significant interaction term (ANOVA, *ddl*=4, F=2.65, *P*<0.05). Indeed, although pre-imaginal development duration increased significantly in three of the four *An. coluzzii* populations from Burkina Faso under ODS conditions (Déou, Oursi and Bama), this

trend was not statistically significant in the Soumousso population nor in the laboratory population from Cameroon (**Fig. 4A**).

Body dry mass was assessed for a total of 220 female mosquitoes (10 to 34 individuals from each experimental group). Analyses showed that the dry mass of female mosquitoes changed significantly between the rearing conditions and that these changes differed among mosquito populations, as shown by the significant interaction term (ANOVA, *ddl*=4, F=31.80, *P*<0.001). Indeed, whereas body dry mass decreased in females from Déou and Oursi (Northern locations) when they were reared in ODS conditions, it increased sharply in females from Bama (Southern location, permanent population; **Fig. 4B**). The trend for decreased dry mass in ODS conditions was not statistically significant in females from Soumousso and Ngousso.

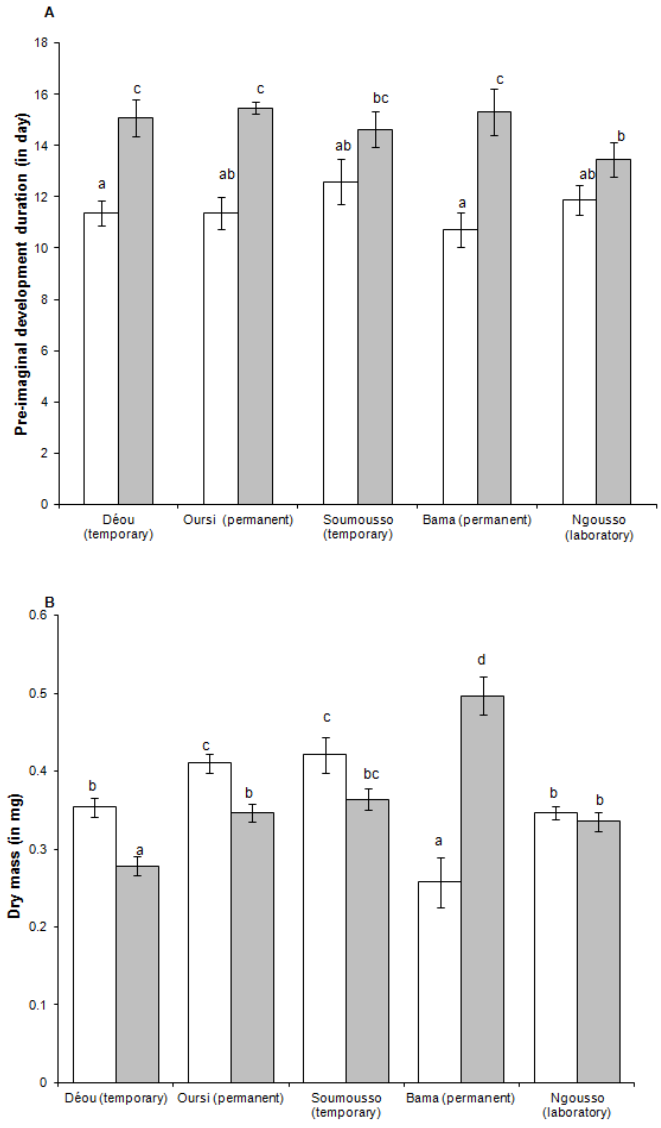

Fig. 4 A/ Mean pre-imaginal development duration (in days) ±SE for the five *An. coluzzii* populations reared under RS (white) and ODS (grey) experimental conditions. B/ Mean body dry mass (in mg) ±SE for the five *An. coluzzii* populations reared under RS (white) and ODS (grey) experimental conditions. Letters in superscript indicate statistical significance at the threshold of α=5%.

3.2. Wing geometric morphometrics

A total of 489 wings from all experimental groups (*i.e.* populations and rearing treatments) were used as template to perform geometric morphometric analyses. Wings were randomly sampled in females issued from the different rearing sessions

3.2.1. Wing size

Significant variation in wing Centroid Size (CS) was found between the two experimental rearing conditions and these changes differed among the five mosquito populations, as supported by a statistically significant interaction term (ANOVA, *d.f.*=1, F= 2.65, *P*<0.05). Although CS increased significantly when mosquitoes were reared under the ODS conditions in females from Oursi, Soumousso, Bama and Ngousso, no such change was observed in those from Déou (Northern location, temporary population; **Fig. 5**).

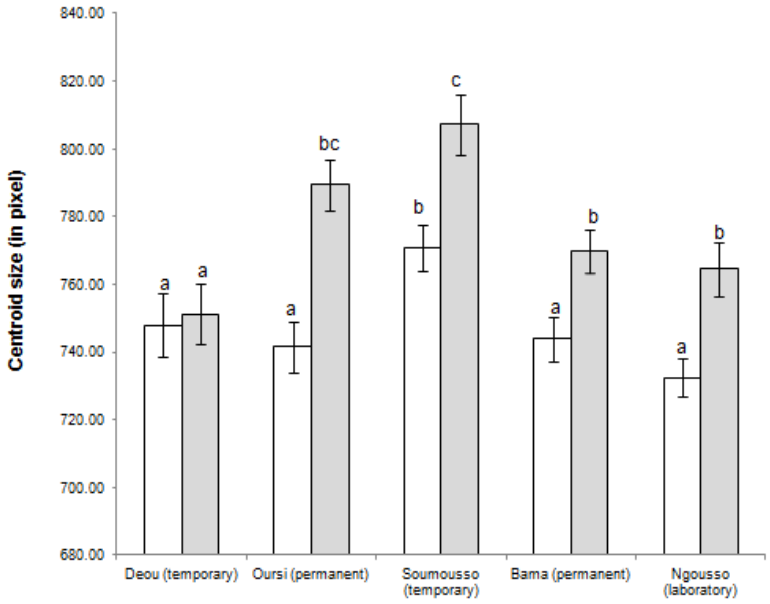

Fig. 5 Mean wing centroid sizes (pixel) ±SE for the five *An. coluzzii* populations reared under RS (white) and ODS (grey) experimental conditions. Letters in superscript indicate statistical significance at the threshold of α=5%.

3.2.2. Wing shape

The MANCOVA analysis showed that wing shape differed significantly between RS and ODS conditions and that these changes differed among the five mosquito populations, as supported by a statistically significant interaction term (MANCOVA, $F_{4,96}$=1.64, *P*<0.001).

A PCA analysis was thus performed to graphically assess the magnitude and direction of shape variation among experimental groups (**Fig. 6**). The first axis of the PCA (PC1) accounted for 45.8% of the total inertia. This axis depicted differences in several components of the wing, including the proximal-distal axis, and the posterior-distal compartment between the Burkina-Faso populations and the laboratory colony originating from Cameroon, and to a lesser extent between the two populations from southern Burkina-Faso (*i.e.* Bama and Soumousso). The second axis (PC2) accounted for 10.3% of the total inertia, and was mainly constructed by shape variation between RS and ODS conditions. Changes along PC2 involved differences in the proximal-posterior compartment, and the anterior-posterior axis of the distal region. In particular ODS-reared mosquitoes sampled from Déou, Oursi, Soumousso and Ngousso showed decreased length of the anterior-posterior axis of wings. A reverse pattern was observed in ODS-reared females An. coluzzii from Bama (**Fig. 6**). Analysis of the neighbour joining tree on the Mahalanobsis distances supported PCA results (**Fig. 7**). Accordingly, wing shape conformation differed more among geographic populations than between experimental rearing conditions within populations.

The Collyer & Adams (2007) procedure performed on wing shape conformation changes between RS and ODS showed that the magnitude of changes did not differ among geographic populations (**Table 1**). However, analyses highlighted significant changes of direction between the two southern populations (i.e. Bama vs Soumousso, θ=100.70°; P<0.001) and between the two permanent ones (i.e. Bama vs Oursi, θ=113.40°; *P*<0.001; **Table 1**).

MANCOVA analysis also highlighted that shape variables were not independent of allometric effects as supported by the significant effect of CS on wing shape variation between experimental rearing conditions (MANCOVA, $F_{4,24}$=1.77, *P*<0.05), and among the five populations (MANCOVA, $F_{4,96}$=1.31, *P*-value<0.05). Accordingly, multivariate regressions performed on mosquito populations showed a significant effect of wing CS on wing shape variation between RS and ODS conditions in females from Soumousso (R^2=0.53, *P*<0.01) and Ngousso (R^2=0.45, *P*<0.001), and their allometric models also differed between RS and ODS (**Table 2**) so that the greater the difference in size, the greater the difference in shape. However, no such relationship was observed in the three other populations.

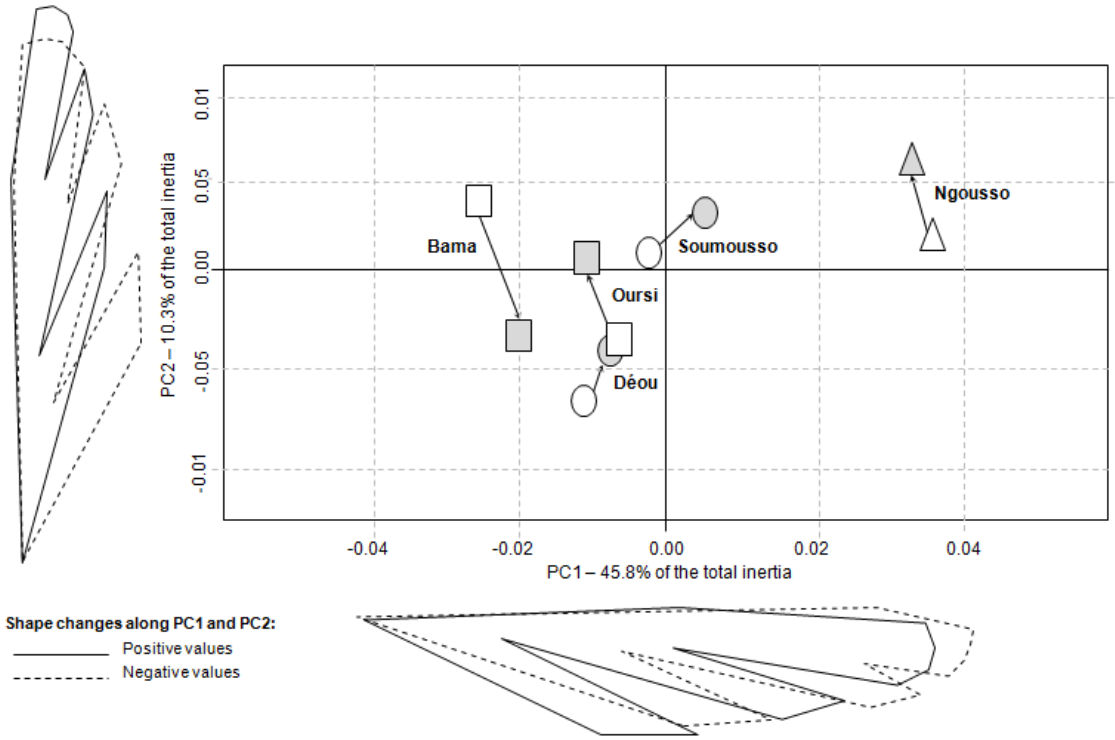

Fig. 6 Projection on the first plane of the Principal Component Analysis (PCA) of 489 individual wings from five *An. coluzzii* populations reared under RS (white) and ODS (grey) conditions. Only the centroids of each group are plotted for ease of interpretation. The direction and magnitude of wing shape variation between RS and ODS are represented by arrows. Both positive and negative mean shape changes along the two first component axes (PC1, PC2) are represented by sketches positioned along each axis, and corresponded to a 3.0 unit of change. Circles represent temporary mosquito populations, squares represent permanent populations, and triangles represent the control laboratory population.

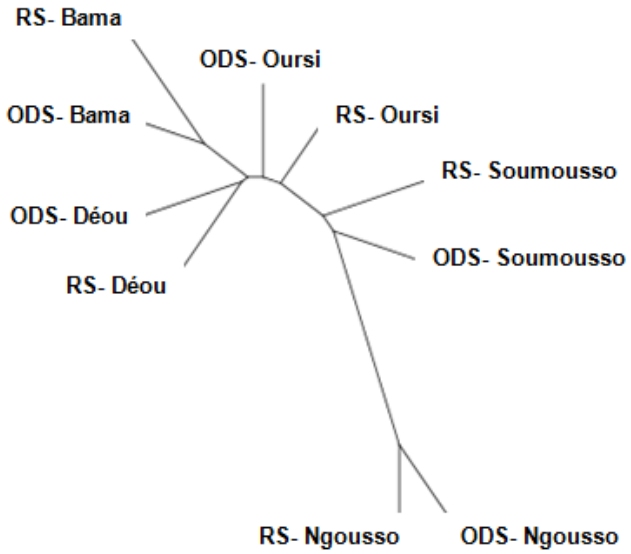

Fig. 7 Neighbour joining tree on the Mahalanobsis distances derived from the wing conformation variables

Table 1. Results of the Collyer & Adams (2007) procedure performed on wing shape conformations of North (*i.e.* Déou and Oursi), South (*i.e.* Soumousso and Bama), permanent (*i.e.* Oursi and Bama) and temporary (*i.e.* Déou and Soumousso) populations of mosquitoes. Both magnitude (ΔD) and direction (θ) of the wing shape variation from RS to ODS are presented with the associated *P*-value (P<0.05). ns= not significant; ***= *P*<0.0001.

Population	Magnitude ΔD	*P*-value	Direction (θ)	*P*-value
North	9.0e-03	ns	60.02	ns
South	2.7e-03	ns	100.70	***
Permanent	4.1e-03	ns	113.4	***
Temporary	4.6e-03	ns	34.36	ns

Table 2. Result of the multivariate regression analysis (column 2) testing the incidence of wing size variation from RS to ODS conditions on the wing shape variations for each *An. coluzzii* population. When a significant allometric incidence is found for a population, a different slopes model analysis (column 3) between RS and ODS rearing conditions is computed. 'ns'= not significant; *= *P*<0.05; **= *P*<0.0.1; ***= *P*<0.0001. 'NA'= Not applicable because of non significant multivariate regression.

Population	Multivariate regression	Different slopes model
Déou	ns (R^2=0.48)	NA
Oursi	ns (R^2=0.22)	NA
Soumousso	** (R^2=0.53)	***
Bama	ns (R^2=0.25)	NA
Ngousso	*** (R^2=0.45)	*

3.2.3. Wing surface area

Analysis showed that the surface area of wings was positively correlated with its size (Pearson test, t_{486}=12, R^2=0.81, *P*<0.001; **Fig. 8**). Therefore, both surface area and wing size presented a similar pattern of variation (**Supplementary data 2**).

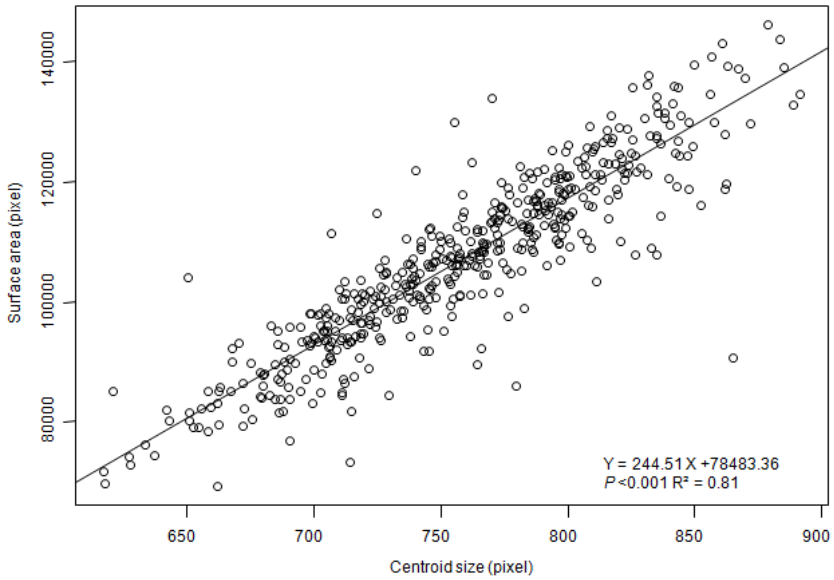

Fig. 8 Linear regression between centroid size (CS) and surface area of mosquito wings.

3.2.4. Metric disparity of the wing conformation

The metric disparity (MD) in wing conformation across all treatments was significantly more important than the MD of each separate ones (**Fig. 9**). Moreover, analysis showed that only females from Ngousso exhibited distinct MD according the experimental rearing conditions. Accordingly, females reared under ODS conditions expressed more diverse wing conformations than their counterparts reared under RS in this test population. Whatever the experimental rearing conditions, females from Bama exhibited more diverse wing conformations (*i.e.* higher MD) than the four other populations.

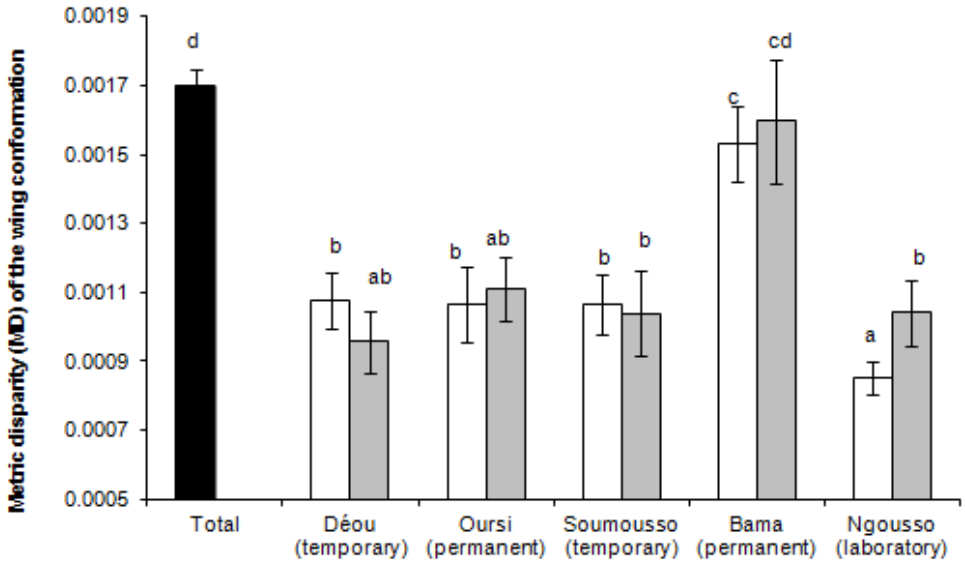

Fig. 9 Metric disparity of the wing shape conformation ±SE for the five *An. coluzzii* populations reared under RS (white) and ODS (grey) experimental conditions. The total metric disparity is presented in the black bar. Letters in superscript indicate statistical significance at the threshold of α=5%.

4. DISCUSSION

The present work provides new information about developmental and associated morphological plasticity in distinct geographic populations of *An. coluzzii* when they are reared under contrasted environmental conditions. Our data mainly support that (i) pre-imaginal development duration changes as a response to the environmental conditions that larvae experience during their development= development is slowed down in all populations under ODS conditions, (ii) body dry mass of emerging

mosquitoes changes according to both the geographic origin of the mosquito population and the environmental conditions they experience during larval development, and (iii) larval rearing conditions and the geographic origin of population further influence wing size, shape and surface area of emerging females. Our work further highlighted the redundancy in information brought by both size and surface area of mosquito wings, as shown by similar patterns of variation and significant correlation among the variables.

In insects, thermal conditions experienced during ontogenesis pilots the duration of larval development. Generally, within a permissive range of temperature, development speeds up with increasing temperature, until an optimal is reached; it then slows down when temperatures increase further (Arbab *et al.*, 2008; Arias *et al.*, 2011; Briere *et al.*, 1999; Damos & Savopoulou-Soultani, 2012; Kjærsgaard *et al.*, 2013). Moreover, examples including the Asian malaria vector *An. stephensi* showed that larval development slows down when temperature fluctuates around hot thermal ranges (Paajimans *et al.*, 2010). Similarly, water temperature in larval breeding sites was shown to influence the duration of larval development in the mosquito *Culex pipiens* (Madder *et al.*, 1983; Rueda *et al.*, 1990) and in major African malaria vectors including *An. funestus* and members of the *An. gambiae* complex (Bayoh and Lindsay, 2004, 2003; Gimonneau *et al.*, 2013; Lyons *et al.*, 2013). Our results are in agreement with these findings and demonstrated significant impact of environmental rearing conditions on *An. coluzzii* pre-imaginal development duration. In particular, the results suggested that mosquitoes exposed to dry season conditions (*i.e.* ODS) with high mean temperature and strong daily fluctuations slowed down pre-imaginal development, suggesting the ODS conditions divert from the optimum thermal development range of this mosquito. Interestingly however, our results showed this effect was not observed in all of our test populations. Effects of thermal fluctuation on the development duration of pre-imaginal stages might be context-specific depending on the proximity of the experimental conditions to the thermal development thresholds of the different mosquito populations (Kingsolver *et al.*, 2009). Moreover, although extended larval development duration in ODS conditions resulted in larger emerging adults (*i.e.* higher wing size) in all our test populations, these adults were generally lighter than those

obtained after rearing under RS conditions, except in the Bama population. This finding suggests that the ability of mosquito larvae to harvest, store and utilise resources is altered under strong thermal fluctuations, as demonstrated in other insect species (Lounibos *et al.*, 2002; Reiskind & Zarrabi, 2012). In *Drosophila* sp. re-allocation of resources under strong temperature fluctuation was explained by increased metabolic demands at the cellular level during development when insects are exposed to higher temperatures (Czarnoleski *et al.*, 2013).

Moreover, the Bama population behaved at odds compared to the other test populations, showing increased body weight when reared under ODS conditions. Maintenance of the dry weight-wing length allometric relationship in Bama as opposed to other *An. coluzzii* geographic populations hence suggests intra-species plasticity in foraging efficiency under fluctuating temperatures and possible shifts in thermal reaction norms between geographic and or permanent *vs.* temporary populations of *An. coluzzii*, as suggested for other Diptera species (Huey *et al.*, 2000; Lyons *et al.*, 2013; Reiskind & Zarrabi, 2012). More collections from a wider area representative of the distribution range of *An. coluzzii* in Africa would be needed to draw strong conclusions about local adaptation to thermal environment in this species.

Interpreting pattern of variation of wing shape conformation is obviously tentative and hypothetic. Indeed, the incidence of geometric morphometric changes of insect's wing in flight aerodynamic remains poorly understood (Debat *et al.*, 2008; Fry *et al.* 2003; Gilchrist *et al.* 2000), and it remains very difficult to connect such geometric variations with functional constraints for organisms. Notwithstanding, there is evidence that the changes we observed in the anterior-posterior axis of the distal compartment of mosquito wings influence the wing aerodynamic lift during flight (Vogel, 1981). Changes in wing size and surface area were also observed as a response to the environmental conditions experienced by mosquitoes during larval development. Accordingly, results showed that both size and surface area of wings, which were highly correlated and therefore redundant, increase in ODS-reared mosquitoes, expect in Déou females, suggesting that these two parameters also depend upon the geographic origin of mosquitoes. One hypothesis to explain such a wing variations could be based on air conditions variations at the onset of the dry season.

Air density (*d*) is known to depend on both ambient temperature and RH conditions ($d = \frac{P}{R.T}$, with P the air pressure, R a constant heat capacity dependent of the RH, and T the ambient temperature). Air density might thus change from one season to the next in dry savannahs of West Africa. Incidences of air density changes on flight performance of insects have been already studied under high altitude (Dillon *et al.*, 2006). Accordingly, authors showed that insects with greater wing area relative to body size decrease the induced velocity required for sustaining flight at high altitude, therefore minimizing the energetic cost of flight in low air density conditions. For instance, the mountain honeybees have longer wings and greater wing surface area but invariant body mass relative to their congenerics from lowlands (Hepburn *et al.*, 1998). We observed similar patterns of variation in female *An. coluzzii* from Oursi, Soumousso and Ngousso when they were reared under ODS conditions. Females increased wing size and surface area but did not change body dry mass. No such pattern was observed in ODS-reared females from Déou. Indeed, these females exhibited a decrease in body dry mass and no change in wing size or surface area. Again, because the population from Déou is present only during the rainy season in the wild, this outcome might result from exposure to stressful conditions during development that are outside of the usual temperature range the species is exposed to in its natural habitat.

In a recent study, Yaro *et al.* (2012) observed a range of phenotypes expressed by Sahelian populations of *An. coluzzii* in Mali with respect to their reproductive behaviour. These authors proposed that local mosquito populations might group together specimens with different aestivation abilities, including “strong aestivators” programmed to engage into a state of diapause (*e.g.* gonotrophic dissociation) at the onset of the dry season, and “weak aestivators” that will depend upon surface water availability to terminate their gonotrophic cycle and lay their eggs. Heterogeneity along similar lines has also been described in overwintering *Culex* mosquitoes in temperate environments (Reisen *et al.*, 2010; Tsuda & Kim, 2008). It is noteworthy in our study, that higher heterogeneity in wing shape conformation and changes thereof between RS and ODS conditions compared to other test populations was demonstrated for the Bama population of *An. coluzzii*, as revealed by the Collyer & Adams (2007)

procedure (Table 1) and our disparity analysis (Figure 9). Such developmental and morphological heterogeneity might reflect the existence of higher phenotypic plasticity and different survival strategies (*e.g.* aestivation abilities) within the local *An. coluzzii* population from Bama. Further studies might shed light on local phenotypic diversity of *An. coluzzii* in such environment where large scale irrigation schemes and surrounding temporary surface water collections offer diverse opportunities for mosquito breeding during the rainy season and expose immature stages to various biotic and abiotic factors that might differentially influence the set-up of alternative dry season survival strategies in adults (Huestis & Lehmann, 2014, Lehmann *et al.*, 2012, 2014).

5. CONCLUSION

Overall results showed that wing size, shape and surface area of female *An. coluzzii* depend upon both the genetic background (geographic origin) and rearing conditions experienced by mosquitoes during their larval development. In particular, results highlighted that wing shape conformation mainly depend on the geographic and climatic conditions of the mosquito's habitat, whereas both size and surface mainly change as a response to the environmental conditions perceived during pre-imaginal development. Further investigations are however needed to understand the functional roles of these geographic and seasonal adjustments and to link such plasticity with the mosquito phenology observed in the fields. Additional work is also required to examine phenotypic plasticity locally, and especially in areas where permanent mosquito breeding is possible in large irrigated areas (*e.g.* Bama) and mosquito phenotypic plasticity is highest.

Acknowledgements

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SUPPLEMENTARY DATA

Supplementary data 1. Methodology and results of the relative geometric morphometric measurement error.

Methodology & Analysis

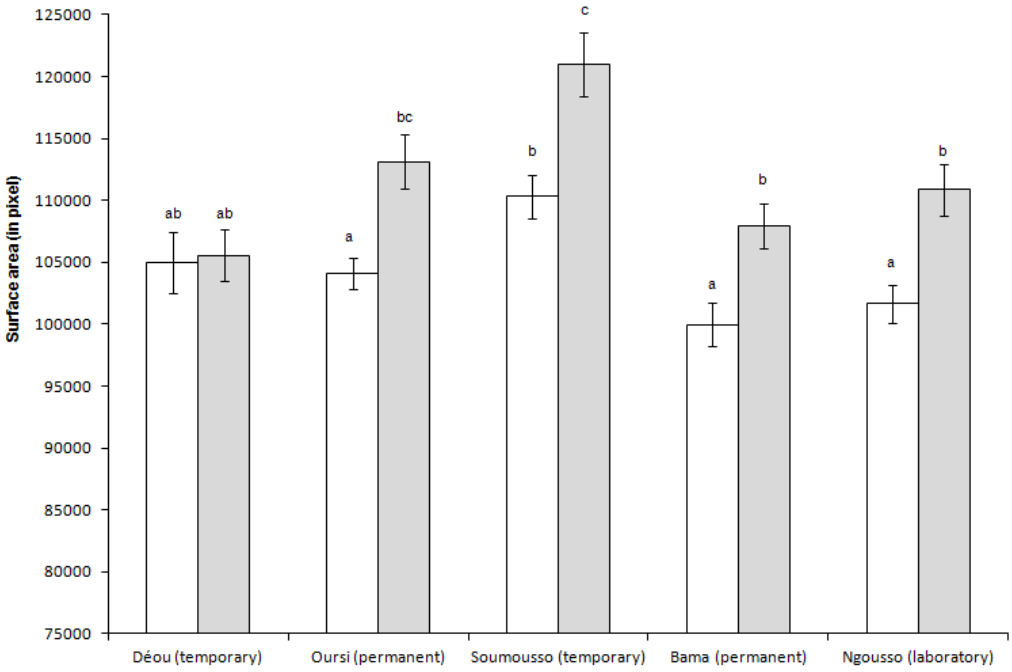
Common errors in Geometric Morphometrics are pictures capture and LM digitisation (Arnqvist & Mårtensson, 1998). To detect such error, a random set of 28 wing picture capture and LM digitisation were repeated twice by the same operator (Alibert *et al.*, 2001). Effect of time between digitisation and capture was also considered by repeating the procedure with 1 month interval. The relative overall measurement error to the biological variation among individual was then calculated using the "individual wing" as the main effect in a procrustes ANOVA on wing size and shape variations. Then, a two-way ANOVA and Tukey *post-hoc* tests were used to assess CS variation between the two experimental conditions (RS, ODS), and among the five mosquito colonies. Based on 10,000 random permutations, a non-parametric procedure was applied to test for statistical significance of wing size variation among experimental groups.

Results

Analysis of the relative measurement error showed no significant effect on both wing size and shape variables. Relative error was 43.00 and 13.19 times lower than inter-individual biological variation in size and shape, respectively (see **table**).

Table. Results of the Procrustes ANOVA computed on individual wings photographed and digitized twice for assessing the Geometric Morphometric measurement error. The *F*-value corresponds to the ratio of inter-individual variance over variation induced by the measurement error. All numeric data are rounded to two decimals. ***= P< 0.0001.

Dataset	Effects	SS	MS	*d.f.*	*F*-value	*P*-value
Centroid size (N= 28*2 = 56 digitisations)	Individual	204625.39	7578.72	27	43.00	***
	Error	4935.29	176.26	28		
Shape (N= 28*2 = 56 digitisations)	Individual	$8.22e^{-02}$	$1.52\ e^{-04}$	540	13.19	***
	Error	$8.80e^{-03}$	$1.57\ e^{-05}$	560		



Supplementary data 2. Barplot of the wing surface area means (in pixel) ±SE of the five mosquito colonies reared under RS (white) or ODS (grey) experimental conditions. Separate letters indicate significant content differences (*P*-value<0.05).

Chapitre IV

Discussion générale et Perspectives

1. **Discussion générale**

2. **Pistes de recherche à court et moyen termes**

1. Discussion générale

L'objectif de ce travail de thèse est d'identifier, déterminer et interpréter les ajustements des caractères écophysiologiques et morphologiques des femelles *An. coluzzii* et *An. gambiae* expérimentalement exposées aux conditions climatiques de la saison des pluies et du début de la saison sèche qu'elles rencontrent dans leur habitat naturel au Burkina-Faso. Nous supposons que les ajustements de ces caractères permettront d'identifier les mécanismes de survie établis par ces espèces au début de la saison sèche. Nos résultats montrent que les femelles *An. coluzzii* et *An. gambiae*, préalablement exposées aux conditions du début de la saison sèche, ont de meilleures capacités de résistance à la dessiccation que leurs congénères exposées aux conditions de la saison des pluies (Article I, II, III). Ces résultats suggèrent que les femelles Anophèles ajustent leurs caractères écophysiologiques et morphologiques au début de la saison sèche. Nous souhaitons analyser ces ajustements et comprendre leurs implications dans les stratégies de survie établies par les femelles pendant la saison sèche. Des ajustements des caractères écophysiologiques et morphologiques ont été mis en évidence par l'ensemble de nos travaux. En revanche, ces ajustements ne sont pas équivalents selon l'espèce considérée. Des différences ont également été observées entre les différentes populations d'*An. coluzzii* (Article II, III, IV). Ces résultats suggèrent donc une variabilité de la plasticité phénotypique des femelles Anophèles au niveau spécifique et populationnel. D'après nos travaux, il semble que ces différences soient liées à l'écologie larvaire des femelles, c'est-à-dire à la présence de collections d'eau permanentes toute au long de l'année (permettant une reproduction continue) ou seulement pendant la saison des pluies (nécessitant la mise en place de réponses rapides au début de la saison sèche). Ces résultats sont d'ailleurs appuyés par les travaux de Lehmann et ses collaborateurs (Laboratory of Malaria and Vector Research, NIAID, NIH ; Huestis & Lehmann, 2014 pour une revue de leur travaux) qui suggèrent différents mécanismes de survie en réponse à la saison sèche des femelles Anophèles selon la nature de leur habitat (présence des collections d'eau).

1.1. Etude de la plasticité phénotypique chez *An. coluzzii*

1.1.1. Stratégie d'estivation des femelles

Les travaux de dynamique et de génétique des populations menés par Lehmann et ses collaborateurs suggèrent une stratégie d'estivation pendant la saison sèche chez les femelles *An. coluzzii* (Adamou *et al.*, 2011 ; Huestis & Lehmann, 2014 ; Lehmann *et al.*, 2010). Cependant, déterminer et identifier un phénotype d'estivation chez ces femelles restent difficile. En effet, à ce jour les mécanismes physiologiques et moléculaires de l'estivation chez les insectes restent mal connus et basés sur des hypothèses établies à partir des connaissances actuelles du processus de diapause hivernale (Denlinger & Armbruster, 2014). Or, les contraintes physiologiques subies par les insectes pendant l'estivation sont différentes de celles subies par leurs congénères en diapause hivernale. Des pressions de sélection distinctes, et par conséquent, des mécanismes physiologiques différents pourraient donc exister entre ces deux processus (Denlinger & Armbruster, 2014 ; Yaro *et al.*, 2012).

Nos résultats supportent l'hypothèse que des mécanismes physiologiques distincts entre l'estivation et la diapause hivernale existent. En effet, dans le but de mettre en évidence un phénotype « estivant » chez les femelles *An. coluzzii* nous avons évalué les variations de l'activité métabolique chez ces femelles au début de la saison sèche et pendant la saison des pluies (Article II). La dépression de l'activité métabolique est connue chez les insectes en diapause hivernale (Hahn & Denlinger 2007, 2011 ; Koštál, 2006 pour des revues). Nous supposons donc que les femelles *An. coluzzii* devraient réduire leur activité métabolique au début de la saison sèche. Cependant, nos résultats ne montrent pas une telle diminution de l'activité métabolique des femelles *An. coluzzii* à l'approche de la saison sèche, mais au contraire une augmentation a été observée (Article II). Ces résultats sont donc contradictoires avec l'hypothèse de dormance estivale (estivation) chez ces femelles pendant la saison sèche. Comme nous le supposons, il est possible que les processus physiologiques de l'estivation chez les femelles *An. coluzzii* ne correspondent pas aux normes de réponses que nous connaissons, à ce jour, dans le cadre de la diapause hivernale, ou que les variations de l'activité métabolique ne sont pas des marqueurs de l'estivation chez ces insectes. Il est également possible que les femelles *An. coluzzii* n'estivent pas pendant la saison sèche, ou encore que les conditions appliquées dans cette étude ne sont pas suffisantes pour initier ou détecter une stratégie d'estivation chez ces femelles. Par exemple, en conditions naturelles, les changements

environnementaux au début de la saison sèche épuisent progressivement les collections d'eau. Ainsi, la densité de larves dans une surface donnée est plus importante que pendant la saison des pluies. Cet effet, nommé effet d'amassement (ou « crowding effect »), pourrait donc constituer un marqueur de stress pour les insectes et induire la stratégie d'estivation des femelles *An. coluzzii*. Par ailleurs, cet effet a pour conséquence de limiter la ressource trophique disponible par individu. Dans nos expérimentations, la densité de larves par bac d'élevage et la quantité de nourriture disponible ont été normalisées afin de ne pas induire de variables supplémentaires aux réponses observées. En effet, nous supposons que les facteurs climatiques sont suffisants pour induire les phénotypes de réponse. Des analyses complémentaires sont toutefois nécessaires pour tester l'influence de l'effet d'amassement sur les réponses des femelles aux changements de l'environnement.

Dans ses études, Huestis *et al.* (2011, 2012) montrent que les femelles *An. coluzzii* prélevées directement sur le terrain, dans la région du Sahel, tout au long de la saison sèche, expriment des profils complexes de variations de l'activité métabolique. Globalement, les femelles montrent une augmentation de l'activité métabolique, comme observée dans nos travaux (Article II). Ainsi, bien que nous n'ayons pas pris en considération l'effet d'amassement, nos résultats semblent illustrer les réponses réellement exprimées par les femelles *An. coluzzii* pendant la saison sèche *in natura*. D'autre part, nos travaux montrent que l'augmentation de l'activité métabolique n'augmente pas la transpiration des insectes au début de la saison sèche (absence de relation entre l'activité métabolique et la transpiration des femelles ; Article II). Ce résultat suggère donc que des mécanismes limitant la transpiration des femelles *An. coluzzii*, autre que l'ajustement de leur activité métabolique, sont mis en place au début de la saison sèche.

1.1.2. Réarrangement biochimique de la cuticule : un marqueur de l'estivation chez *An. coluzzii* ?

Des réarrangements biochimiques susceptibles d'impacter la perméabilité de la cuticule des moustiques ont été observés chez les femelles d'*An coluzzi* (Article I). En effet, une heure seulement après leur émergence, les femelles *An. coluzzii* exposées aux conditions du début de la saison sèche montrent une plus intense accumulation de

protéines du type RR-2 et d'acides aminés (ex. valine, phénylalanine, tyrosine et histidine ; Article I) connus pour participer aux processus de sclérotisation et de mélanisation de la cuticule chez les insectes et ainsi participer à la synthèse d'une cuticule plus rigide et hydrophobe (Andersen, 1979 ; Behmer & Joern, 1993 ; Benoit *et al.*, 2010a ; Karouzou *et al.*, 2007). L'ajustement de la perméabilité de la cuticule est connu pour influencer la transpiration des insectes (Gibbs & Rajpurohit, 2010; Nelson & Lee, 2004 ; Wagoner *et al.*, 2014). L'ajustement de la composition biochimique de la cuticule des femelles *An. coluzzii* constituerait donc une réponse des femelles pour améliorer leurs capacités de résistance à la dessiccation. D'ailleurs nos résultats, montrent que les femelles *An. coluzzii*, préalablement exposées aux conditions environnementales du début de la saison sèche, montrent un meilleur taux de survie et un meilleur maintien de leur teneur en eau corporelle en condition très aride (5-7% d'humidité relative et 27 °C) que leurs congénères élevés en conditions de saison des pluies (Article I). L'ajustement de la perméabilité de la cuticule des insectes est bien documenté pendant la diapause (Benoit & Denlinger, 2007 ; Li & Denlinger, 2009). Ainsi, à l'instar de la diapause hivernale, les ajustements de la composition biochimique de la cuticule pourraient constituer un marqueur de l'estivation des femelles *An. coluzzii*. Des analyses complémentaires sont cependant nécessaires pour tester si ces ajustements biochimiques sont constitutifs d'une stratégie de dormance estivale, mais il semble que ces ajustements soient plus marqués chez *An. coluzzii* au début de la saison sèche et sont donc spécifiques aux mécanismes de survie établis par cette espèce (Article I). D'autres paramètres sont connus pour assurer l'imperméabilité de la cuticule, notamment avant l'entrée en diapause. Par exemple, le papillon *Antheraea yamamai* sur-exprime l'enzyme CYP4G, responsable de la synthèse d'hydrocarbones cuticulaires, quelques heures seulement avant l'entrée en diapause hivernale (Yang *et al.*, 2008). Nous supposons que l'ajustement de la composition lipidique de la cuticule des femelles *An. coluzzii*, notamment l'accumulation de longues chaines d'hydrocarbones saturées (n-alkanes), doit être observée chez les femelles exposées aux conditions déshydratantes de la saison sèche (Wagonner *et al.*, 2014). L'analyse de tels ajustements saisonniers chez les femelles Anophèles est actuellement en préparation. Nous espérons ainsi mettre en évidence des marqueurs

lipidiques spécifiques à la saison sèche et impliqués dans l'imperméabilité de la cuticule chez ces femelles.

1.1.3. Variabilité de la plasticité phénotypique chez *An. coluzzii*

Nos résultats montrent des ajustements différents au niveau de l'allométrie des femelles *An. coluzzii* (taille, masse, rapport surface/volume) selon les conditions environnementales perçues. De plus, ces ajustements diffèrent selon l'origine géographique des femelles, et notamment selon qu'elles exploitent des collections d'eau permanentes ou présentes seulement pendant la saison des pluies (Article II, IV). Par exemple, tout au long de l'année, les femelles issues de gîtes permanents sont plus grandes et fines, alors que celles issues de collections d'eau temporaires sont plus petites et grosses (Article II). Ces résultats suggèrent que selon leur origine géographique, les populations d'*An. coluzzii* exploitent, stockent et utilisent différemment leur réserves énergétiques. Par ailleurs, la nature des réserves corporelles utilisées par les femelles *An. coluzzii* et l'expression des gènes codant la synthèse des hormones adipokinétiques, en réponses aux conditions de la saison sèche, diffèrent d'une population à l'autre (Article II, III). Bien que l'ajustement de ces caractères varie selon la population d'*An. coluzzii* considérée, ils semblent que ces ajustements participent à améliorer la valeur adaptative des femelles, à en juger par l'amélioration des capacités de résistance à la dessiccation des différentes populations (Article II, III). Nous supposons donc que cette variabilité de la plasticité phénotypique des femelles *An. coluzzii* doit refléter des mécanismes d'adaptation/acclimatation des femelles à leur environnement. Si tel est le cas, alors nous suggérons que des changements phénotypiques différents entre ces populations doivent être également observés à d'autres niveaux d'analyse.

Nous avons examiné les ajustements du temps de développement et de la géo-morphométrie des ailes des femelles *An. coluzzii* exposées à des conditions contrastées reproduisant la saison des pluies et le début de la saison sèche (Article IV). Ces deux paramètres sont connus pour être de bons marqueurs du niveau d'adaptation locale des insectes à leur environnement. En effet, les variations du temps de développement et de la forme et la taille des ailes des insectes sont pilotées par les variations des paramètres environnementaux (Atkinson, 1994; Couret *et al.*, 2014; Czarnoleski *et al.*,

2013; Kingsolver *et al.*, 2009; Pétavy *et al.*, 2004). Les variations de ces paramètres au sein des populations d'une même espèce suggèrent une adaptation/acclimatation des individus aux conditions locales de leurs environnements. Nos résultats confirment l'hypothèse d'une variabilité de la plasticité phénotypique des femelles *An. coluzzii* au niveau populationnel. En effet, le temps de développement des femelles et la taille et la forme de leurs ailes changent selon les conditions environnementales perçues pendant le développement, mais aussi selon l'origine géographique des populations d'*An. coluzzii* (Article IV).

Une récente étude suggère que l'hétérogénéité des phénotypes de réponses des femelles *An. coluzzii* aux conditions de la saison sèche correspond à des capacités d'estivation distinctes entre populations (Yaro *et al.*, 2012). D'après cette étude, les moustiques peuvent être des « strong aestivator » programmés pour entrer dans un état de dormance dès le début de la saison sèche, ou des formes quiescentes[1] définies comme des « weak aestivator » et dont l'induction de l'état de dormance dépend de la disponibilité des collections d'eau dans l'environnement. Ces derniers entrent dans un état d'estivation seulement lorsque les collections d'eau ne sont plus disponibles dans l'environnement. La variabilité des phénotypes observés au niveau populationnel, chez les femelles *An. coluzzii*, à l'approche de la saison sèche, pourraient donc refléter une variabilité des capacités d'estivation de ces femelles selon les caractéristiques écologiques de leur habitat. Des études complémentaires sont cependant nécessaires pour confirmer cette hypothèse. Dans un premier temps, il est nécessaire de comprendre qu'elles sont les facteurs influençant les réponses physiologiques et morphologiques des femelles au début de la saison sèche. S'agit-il principalement de la disponibilité des collections d'eau pendant la saison sèche comme le suggère Yaro *et al.* (2012) ? Nos résultats semblent suggérer que d'autres paramètres, tel que la sévérité de la saison sèche au niveau local (nord *versus* sud) est également impliqué dans cette variabilité des réponses des femelles Anophèles aux conditions d'élevage. Par exemple, les profils métaboliques des femelles *An. coluzzii* diffèrent selon que les femelles exploitent des collections d'eau permanentes ou temporaires. Cependant cette distinction est plus marquée dans les populations du sud du Burkina-Faso où la sévérité de la saison sèche est moins importante que dans le nord (Article III).

1 La quiescence dépend exclusivement des conditions environnementales. Elle représente une réponse immédiate aux déclins des conditions du milieu, et sans mécanisme de régulation endogène. Ainsi l'arrêt de la quiescence correspond au retour des conditions environnementales favorables

1.2. Etude de la plasticité phénotypique chez *An. gambiae*

A ce jour, l'état de nos connaissances concernant les mécanismes de survie des femelles *An. gambiae* sont dérisoires en dépit de l'importance de ces insectes en épidémiologie (Huestis & Lehmann, 2014). Les analyses de dynamique et de génétique des populations montrent une disparition locale de ces femelles pendant la saison sèche et une recolonisation du milieu au début de la saison des pluies par migration d'individus provenant de zones de refuges (habitats ou micro-habitats plus favorables à leur développement ; Adamaou *et al.*, 2012 ; Huestis & Lehman, 2014 ; Lehmann *et al.*, 2010 ; 2014). Cette hypothèse suggère que ces femelles doivent donc rechercher activement un nouvel habitat au début de la saison sèche.

Nos travaux permettent d'analyser expérimentalement comment les populations d'*An. gambiae* réagissent au début de la saison sèche, et notamment comment les femelles ajustent leur caractères physiologiques. Bien que notre méthodologie ne nous permette pas de déterminer clairement un comportement de migration, nous pouvons néanmoins identifier les marqueurs écophysiologiques de cette stratégie. Nos résultats ont permis d'identifier deux marqueurs biochimiques impliqués dans le métabolisme énergétique des muscles striés alaires (Article I, II) : la proline et l'hormone adipkonétique, l'Anoga-AKH-II. La proline est un acide aminé essentiel au vol des diptères (Scaraffia & Wells, 2003). En effet, la proline alimente le cycle de Krebs et produit le substrat énergétique nécessaire à la contraction des muscles striés (**Fig. 22**). Par exemple, une augmentation de la teneur en proline dans l'hémolymphe des moustiques *Aedes vexans* (Diptera, Culicidae) accroit significativement l'endurance en vol des spécimens (Briegle *et al.*, 2001). L'accumulation de cet acide aminé, au début de la saison sèche, chez *An. gambiae*, suggère donc des besoins énergétiques plus importants au niveau des muscles alaires. La synthèse de la proline et son transport du corps gras à l'hémolymphe puis aux muscles alaires sont assurées par les hormones adipkonétiques (Gäde, 2004; Isabel *et al.*, 2005; Wilps & Gäde, 1990; Ziegler *et al.*, 2011 ; **Fig. 22**). L'implication de ces hormones dans le maintien du vol sur de longues distances à été largement démontrée chez les insectes terrestres (Arrese & Soulages, 2010 ; Van der Horst & Rodenburg, 2012). En effet, ces hormones permettent d'alimenter les muscles alaires des insectes en divers substrats énergétiques (**Fig. 22**). Outre le transport de la proline, les

hormones adipkonétiques assurent également la mobilisation et le transport du tréhalose et du diacylglycerol, deux sources d'énergies majeures impliquées dans la contraction des muscles striés alaires chez les insectes (**Fig. 22**). Des analyses complémentaires restent cependant nécessaires afin de tester si l'accumulation de la proline et de l'Anoga-AKH-II, chez les femelles *An. gambiae* au début de la saison sèche, participe à la synthèse du substrat énergétique indispensable à la contraction des muscles alaires. En effet, notre méthodologie explore les changements physiologiques de l'ensemble du corps des moustiques. Or, des dosages ciblés et isolés de ces composés au niveau des muscles alaires permettraient de confirmer l'hypothèse de l'implication de ces composés dans la contraction des muscles striés alaires. Des dosages d'autres métabolites ou d'enzymes impliqués dans le métabolisme énergétique des muscles alaires (voir **Fig. 22**) permettraient également de rendre compte de l'activité métabolique au sein de ces muscles.

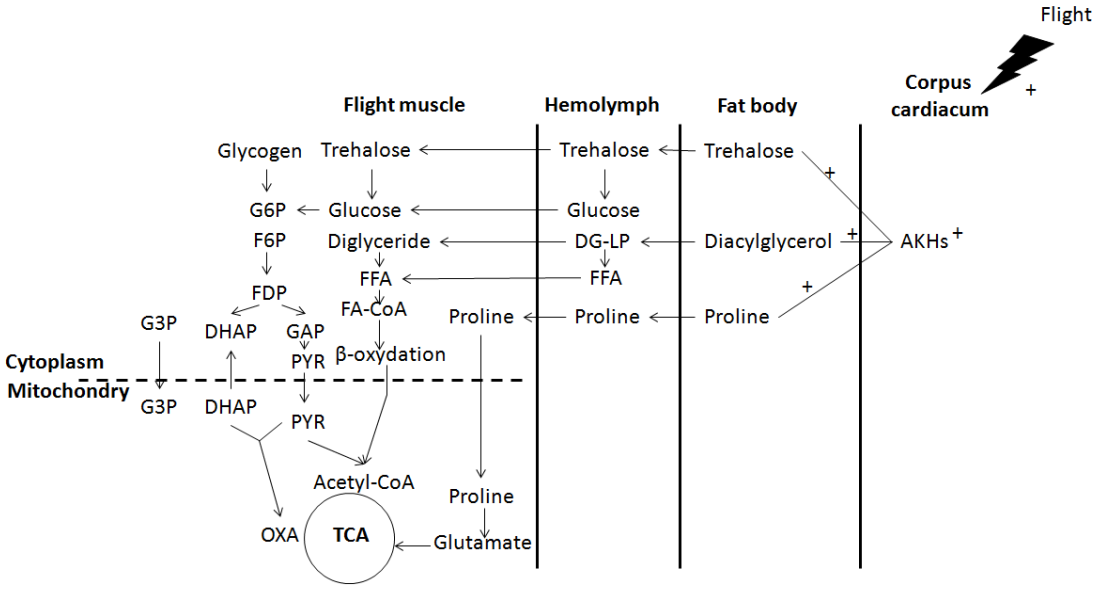


Fig. 22 Construction synthétique de l'incidence de la synthèse des hormones adipokinétiques (AKH) sur les voies métaboliques de la proline, du diacylglycerol et du tréhalose dans le corps gras, l'hémolymphe et les muscles alaires des insectes. Acetyl-CoA = acetyl-coenzyme A, DG-LP = lipoprotein-bound diacylglycerol, DHAP = dehydroxyacetone phosphate, F6P = fructose-6-phosphate, FDP = fructose bisphosphate, FA-CoA = fatty acid-coenzyme A, FFA = free fatty acids, G3P = glycerol-3-phosphate, G6P glucose-6-phosphate, GAP = glyceraldehyde-3-phosphate, OXA = oxaloacetate, PYR = pyruvate. TCA = cycle de Krebs.

Par ailleurs, nos travaux montrent une intense expression de métabolites (ex. proline) et d'enzymes (ex. isocitrate dehydrogénase, citrate synthase) impliqués dans le métabolisme énergétique (cycle de Krebs) chez *An. gambiae*, quelque soit les

conditions d'élevage (Article I). De plus, cette sur-expression est significativement plus importante que chez les femelles *An. coluzzii*, D'autre part, les femelles *An. gambiae* montrent une activité métabolique élevée tant en saison des pluies qu'au début de la saison sèche (Article II). Ces résultats suggèrent que les femelles *An. gambiae* ont un niveau d'activité métabolique, et notamment du métabolisme énergétique, aussi élevé au début de la saison sèche que pendant la saison des pluies. A ce jour, nos travaux ne nous permettent pas de savoir si ces ajustements sont induits en réponse aux conditions stressantes de la saison sèche (ex. métabolisme du stress, synthèse d'osmoprotecteurs, etc.) ou dans le but de produire le substrat énergétique nécessaire à la contraction des muscles alaires.

2. Pistes de recherche à court et moyen termes

2.1. Evaluer le degré de perméabilité de la cuticule

Nos travaux ont mis en lumière des ajustements biochimiques et métaboliques chez les femelles *An. coluzzii* et *An. gambiae* à l'approche de la saison sèche. Ces travaux suggèrent des ajustements au niveau de la composition biochimique de la cuticule de ces femelles (accumulation de protéines cuticulaires de type RR-2, valine, phénylalanine, etc. ; Article I). Nous supposons que ces ajustements permettent une meilleure rigidité et perméabilité de la cuticule des femelles pendant la période contraignante de la saison sèche. Nous souhaitons donc vérifier cette hypothèse grâce à des analyses comparatives de coupes de cuticule des femelles Anophèles exposées aux conditions de la saison des pluies et du début de la saison sèche par microscopie électronique à balayage (MEB). Nous supposons que les femelles montreront des différences au niveau de l'épaisseur et de la structuration des différentes couches cuticulaires selon les conditions d'élevage. Par exemple une épaisseur plus importante de la procuticule (sous-couche sclérotisée de la cuticule) est attendue chez les femelles exposées aux conditions du début de la saison sèche. En effet, la procuticule est connue pour assurer la rigidité de la cuticule chez les insectes. Or, nous supposons qu'une cuticule plus rigide est nécessaire pour améliorer la résistance à la dessiccation des femelles Anophèles au début de la saison sèche.

Par ailleurs, la perméabilité de la cuticule est essentiellement associée à la composition lipidique de la couche la plus externe (l'épicuticule). Une accumulation de longues chaines d'hydrocarbones saturées est connus pour améliorer l'imperméabilité de la cuticule (Gibbs *et al.*, 1998 ; Hadley, 1994). Une analyse par chromatographie à phase gazeuse couplée à un spectrophotomètre de masse (GC-MS) de la composition lipidique de cette couche chez les femelles Anophèles exposées aux deux conditions d'élevage (saison des pluies et début de la saison sèche) permettrait de renseigner sur le degré de perméabilité de la cuticule des femelles d'une saison à l'autre. D'autre part, nous supposons que l'établissement d'une cuticule imperméable et fortement hydrophobe doit également avoir une influence sur la sensibilité des femelles Anophèles à la pulvérisation d'insecticides utilisée pour leur gestion. Déterminer la composition biochimique de la cuticule est donc d'autant plus intéressant qu'elle renseignera également sur la perméabilité de la cuticule aux insecticides et donc de l'efficacité des méthodes de gestions actuelles.

2.2. Tester expérimentalement l'hypothèse de migration chez *An. gambiae*

Nos travaux suggèrent une meilleure propension à disperser chez les femelles *An. gambiae* au début de la saison sèche. Deux potentiels marqueurs biochimiques des stratégies de migration ont été mis en évidence. Des analyses expérimentales complémentaires peuvent cependant être effectuées afin de confirmer cette hypothèse. Par exemple, analyser l'endurance du vol des femelles dans des tunnels de vol et/ou la nature et la quantité des réserves énergétiques stockées par les femelles *An. gambiae*, au début de la saison sèche et pendant la saison des pluies, permettraient de rendre compte d'une meilleure capacité des femelles à disperser ou non. Par ailleurs, il est admis que les phénotypes « migrateur » montrent un polymorphisme musculaire au niveau des muscles alaires. En effet, les générations migratrices d'insectes montrent, par exemple, des muscles dorso-alaires plus développés que les générations non migratrices (Socha & Šula, 2006 ; Zera & Denno, 1997 ; **Fig. 23**). L'exploration d'un tel polymorphisme permettrait d'identifier des femelles potentiellement « migrantes » chez *An. gambiae* à l'approche de la saison sèche et pendant de la saison des pluies.

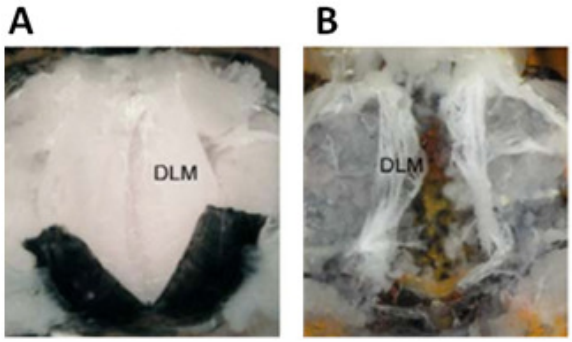


Fig. 23 Photographies des muscles alaires dorso-longitudinaux (DLM) chez *Pyrrhocoris apterus* 5 jours après l'émergence. A-Muscles alaires développés des phénotypes « migrants » ; B- Muscles alaires non développés par des phénotypes « non-migrants ». D'après Socha & Šula, 2006.

2.3. Profilage moléculaire

Nos analyses de profilages métabolomiques et protéomiques ont mis en évidence peu de marqueurs des mécanismes de survie des femelles Anophèles aux conditions contraignantes de la saison sèche (Article I, II, III). De plus, certains de ces marqueurs peuvent être impliqués dans divers processus métaboliques (ex. l'accumulation de la proline chez *An. gambiae*). Il apparait donc difficile d'interpréter ces changements et d'identifier les réels mécanismes de survie établis par ces insectes. Nous supposons que des analyses de profilages moléculaires exploratoires (ex. transcriptomique comparative) des femelles *An. coluzzii* et *An. gambiae* expérimentalement exposées à des conditions reproduisant la saison des pluies et la saison sèche permettront (1) d'identifier un plus grand nombre de marqueurs des mécanismes de survie des insectes et (2) de déterminer les voies de régulation métabolique sur-exprimées par les femelles. Ces données couplées à nos données de métabolomiques et de protéomiques permettront d'accéder à une meilleure compréhension des mécanismes de survie des femelles à différentes échelles d'analyse. Ces analyses sont d'autant plus pertinentes que le génome des moustiques *An. gambiae* est entièrement séquencé (Holt et al. 2002) et qu'un grand nombre de gènes ont déjà été identifiés.

2.4. Induction des mécanismes de survie: période(s) et facteur(s) clés

L'induction des mécanismes de survie des femelles *An coluzzii* et *An. gambiae* aux conditions stressantes de la saison sèche a été observée dès l'émergence des imagos (Article I). Ces résultats suggèrent que la perception des changements de l'environnement a lieu pendant le développement pré-imaginal et/ou à la génération précédente (message transgénérationel d'origine maternel et/ou paternel). Dans un premier temps, il serait donc intéressant de déterminer la période critique du

développement des Anophèles impliquée dans l'induction des mécanismes de survie à la saison sèche. Des expérimentations comparatives des phénotypes écophysiologiques des femelles exposées à des conditions d'élevages contrastées (saison des pluies et saison sèche) à différents moments de leur développement permettraient d'identifier une(des) période(s) clé(s) dans l'induction des mécanismes de survie de ces insectes. Ces expérimentations pourront inclure des comparaisons transgénérationelles en exposant, par exemple, les parents à des conditions d'élevages distinctes et en observant les phénotypes de leurs progénitures.

Dans un second temps, une fois la(les) période(s) clé(s) de l'induction des mécanismes de survie des femelles Anophèles déterminée(s), découpler les paramètres abiotiques susceptibles d'induire la perception des changements environnementaux pendant cette(ces) période(s) permettraient d'identifier le(les) facteur(s) impliqué(s) dans l'induction des mécanismes de survie. A terme, ces connaissances permettront une meilleure compréhension de la survie des femelles Anophèles dans leur milieu et de réadapter les méthodes de contrôles à des moments clés du développement des insectes (par exemple avant l'induction des mécanismes de survie, au début de la saison sèche).

2.5. La prise de sang peut-elle améliorer la résistance des femelles Anophèles aux conditions de la saison sèche ?

Les analyses expérimentales menées sur la physiologie de la résistance à la saison sèche des moustiques vecteurs du paludisme examinent principalement des individus nourris à base de solutions sucrées (Article II, III; Gray *et al.*, 2009). Cependant, les femelles moustiques sont hématophages et se nourrissent tant de jus sucrés que de sang. En effet, la prise de sang représente une ressource trophique primordiale pour la reproduction des femelles. Les composés contenus dans le sang initient la synthèse des protéines impliquées dans la vitellogenèse des insectes (Ahmed *et al.,* 2001) et leur oogenèse (Fiil, 1976). Néanmoins, le sang doit également être considéré comme une source non négligeable d'eau et de nutriments pour les organismes exposés aux conditions déshydratantes de la saison sèche (Benoit *et al.*, 2011; 2007; Benoit & Denlinger, 2007). Par ailleurs, les récents travaux de Yaro *et al.* (2012) suggèrent que les femelles *An. coluzzii* ne modifient pas leur comportement d'alimentation en sang

d'une saison à l'autre, mais que l'allocation de cette ressource dans la reproduction varie selon l'environnement des moustiques (présence de collections d'eau pour la ponte des œufs). Ces résultats suggèrent alors que l'allocation de la ressource trophique dans les différentes fonctions biologiques des femelles, dépend des conditions environnementales perçues par les femelles. Nous supposons donc que les nutriments et l'eau contenus dans le sang humain permettraient aux femelles Anophèles d'améliorer leurs capacités de résistance et de tolérance à la dessiccation au début de la saison sèche. Une analyse est actuellement en cours afin d'évaluer la contribution du sang sur les processus de résistance à la dessiccation chez différentes populations de femelles *An. coluzzii* exposées depuis le stade embryonnaire aux conditions climatiques de la saison des pluies (RS) et du début de la saison sèche (ODS).

2.6. La présence d'un parasite du genre *Plasmodium* altère-t-elle les mécanismes de réponses des femelles Anophèles aux conditions de la saison sèche ?

Nos travaux illustrent les réponses de femelles moustiques saines, c'est-à-dire non parasitées par les *Plasmodium* responsables du paludisme. Cependant, la présence d'un parasite au sein d'un organisme peut modifier son comportement, son écologie, sa biologie et sa physiologie (Aboagye-Antwi *et al.*, 2010 ; Bourtzis & Miller, 2008 ; Dedeine *et al.*, 2001). Nous supposons que les femelles *An. coluzzii* et *An. gambiae* présentent des réponses aux conditions de la saison sèche différentes selon qu'elles sont ou non parasitées. Il apparait nécessaire de tester si les capacités de résistance et de tolérance à la dessiccation sont également influencées par la présence du *Plasmodium* au sein des femelles. Par ailleurs, de récents travaux suggèrent que les capacités de survie des femelles *An. gambiae* à un stress hydrique sont différemment affectées selon que les femelles sont infectées par des oocytes ou des sporozoïtes (Aboagye-Antwi *et al.*, 2010). Le stade de développement des parasitent semblent donc impacter différemment la physiologie de leur hôte. Ce paramètre devrait donc être pris en considération dans nos futures analyses.

Références

A

Aboagye-Antwi, F., Tripet, F., 2010. Effects of larval growth condition and water availability on desiccation resistance and its physiological basis in adult Anopheles gambiae sensu stricto. Malar. J. 9, 225.

Adamou, A., Dao, A., Timbine, S., Kassogué, Y., Yaro, A.S., Diallo, M., Traoré, S.F., Huestis, D.L., Lehmann, T., 2011. The contribution of aestivating mosquitoes to the persistence of Anopheles gambiae in the Sahel. Malaria J, 10, 151. doi:10.1186/1475-2875-10-151

Addo-Bediako, A., Chown, S.L., Gaston, K.J., 2000. Thermal tolerance, climatic variability and latitude. Proc. R. Soc. Lond. B Biol. Sci. 267, 739–745. doi:10.1098/rspb.2000.1065

Addo-Bediako, A., Chown, S.L., Gaston, K.J., 2001. Revisiting water loss in insects: a large scale view. J. Insect Physiol. 47, 1377–1388. doi:10.1016/S0022-1910(01)00128-7

Ahearn, G.A., 1970. The control of water loss in desert tenebrionid beetles. J. Exp. Biol. 53, 573–595.

Ahmed, A.M., Maingon, R., Romans, P., Hurd, H., 2001. Effects of malaria infection on vitellogenesis in Anopheles gambiae during two gonotrophic cycles. Insect Mol. Biol. 10, 347–356. doi:10.1046/j.0962-1075.2001.00273.x

Ahmed, A.M., Baggott, S.L., Maingon, R., Hurd, H., 2002. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito Anopheles gambiae. Oikos 97, 371–377. doi:10.1034/j.1600-0706.2002.970307.x

Ananthakrishnan, T.N., Whitman, D., 2005. Insect phenotypic plasticity: diversity of responses. 213 pp.

Andersen, S.O., 1979. Biochemistry of insect cuticle. Annu Rev Entomol, 24, 29–59. doi: 10.1146/annurev.en.24.010179.000333

Andersen, D.H., Pertoldi, C., Scali, V., Loeschcke, V., 2005. Heat stress and age induced maternal effects on wing size and shape in parthenogenetic Drosophila mercatorum. J. Evol. Biol. 18, 884–892. doi:10.1111/j.1420-9101.2005.00955.x

Arbab, A., Kontodimas, D.S., Mcneill, M.R., 2008. Modeling embryo development of Sitona discoideus Gyllenhal (Coleoptera: Curculionidae) under constant temperature. Environ. Entomol. vol. 37, no. 6, pp. 1381–1388.

Arias, M.B., Poupin, M.J., Lardies, M.A., 2011. Plasticity of life-cycle, physiological thermal traits and Hsp70 gene expression in an insect along the ontogeny: Effect of temperature variability. J. Therm. Biol. 36, 355-362.

Arrese, E.L., Soulages, J.L., 2010. Insect fat body: energy, metabolism, and regulation. Annu. Rev. Entomol. 55, 207–225. doi:10.1146/annurev-ento-112408-085356

Atkinson, D., 1994. Temperature and organism size - a biological law for ectotherms? Advances in Ecological Research. 25,1-58.

Ayala, D., Caro-Riaño, H., Dujardin, J.-P., Rahola, N., Simard, F., Fontenille, D., 2011. Chromosomal and environmental determinants of morphometric variation in natural populations of the malaria vector Anopheles funestus in Cameroon. Inf. Gen. and Evol. 11, 940–947. doi:10.1016/j.meegid.2011.03.003

B

Baldet, T., Diabaté, A., Guiguemdé, T.R., 2003. Malaria transmission in 1999 in the rice field area of the Kou Valley (Bama), (Burkina Faso). Santé Montrouge Fr. 13, 55–60.

Bardel, J., Louwagie, M., Jaquinod, M., Jourdain, A., Luche, S., Rabilloud, T., Macherel, D., Garin, J., Bourguignon, J., 2002. A survey of the plant mitochondrial proteome in relation to development. Proteomics 2, 880–898. doi:10.1002/1615-9861(200207)2:7

Baylac, M., Daufresne, T., 1996. Wing venation variability in Monarthropalpus buxi (Diptera, Cecidomyiidae) and the quaternary coevolution of box (Buxus sempervirens L.) and its midge, in: Marcus, L.F., Corti, M., Loy, A., Naylor, G.J.P., Slice, D.E. (Eds.), Advances in morphometrics (pp. 285–301). New York: Plenum Press.

Bayley, M., Holmstrup, M., 1999. Water vapor absorption in arthropods by accumulation of myoinositol and glucose. Science 285, 1909–1911.

Bayoh, M.N., Lindsay, S.W., 2003. Effect of temperature on the development of the aquatic stages of Anopheles gambiae sensu stricto (Diptera: Culicidae). Bull. Entomol. Res. 93, 375–381. doi:10.1079/BER2003259

Bayoh, M.N., Lindsay, S.W., 2004. Temperature-related duration of aquatic stages of the Afrotropical malaria vector mosquito Anopheles gambiae in the laboratory. Med. Vet. Entomol. 18, 174–179. doi:10.1111/j.0269-283X.2004.00495.x

Behmer, S.T., Joern, A., 1993. Dietary selection by the generalist grasshopper Phoetaliotes nebrascensis (Orthoptera: Acrididae). Funct Ecol, 7, 522–527.

Beier, J.C., Perkins, P.V., Onyango, F.K., Gargan, T.P., Oster, C.N., Whitmire, R.E., Koech, D.K., Roberts, C.R., 1990. Characterization of Malaria Transmission by Anopheles (Diptera: Culicidae) in Western Kenya in Preparation for Malaria Vaccine Trials. J. Med. Entomol. 27, 570–577.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B, 57, 289–300.

Benoit, J.B., Yoder, J.A., Rellinger, E.J., Ark, J.T., Keeney, G.D., 2005. Prolonged maintenance of water balance by adult females of the American spider beetle, Mezium affine Boieldieu, in the absence of food and water resources. J. Insect Physiol. 51, 565–573. doi:10.1016/j.jinsphys.2005.03.001

Benoit, J.B., Del Grosso, N.A., Yoder, J.A., Denlinger, D.L., 2007. Resistance to dehydration between bouts of blood feeding in the bed bug, Cimex lectularius, is enhanced by water conservation, aggregation, and quiescence. Am. J. Trop. Med. Hyg. 76, 987–993.

Benoit, J.B., Denlinger, D.L., 2007. Suppression of water loss during adult diapause in the northern house mosquito, Culex pipiens. J. Exp. Biol. 210, 217–226. doi:10.1242/jeb.02630

Benoit, J.B., 2010. Water management by dormant insects: comparisons between dehydration resistance during summer aestivation and winter diapause. Aestivation 209–229.

Benoit, J.B., Patrick, K.R., Desai, K., Hardesty, J.J., Krause, T.B., Denlinger, D.L., 2010a. Repeated bouts of dehydration deplete nutrient reserves and reduce egg production in the mosquito Culex pipiens. J Exp Biol, 213, 2763–2769. doi: 10.1242/jeb.044883

Benoit, J.B., Lopez-Martinez, G., Phillips, Z.P., Patrick, K.R., Denlinger, D.L., 2010b. Heat shock proteins contribute to mosquito dehydration tolerance. J. Insect Physiol. 56, 151–156. doi:10.1016/j.jinsphys.2009.09.012

Benoit, J.B., 2011. Stress Tolerance of Bed Bugs: A Review of Factors That Cause Trauma to Cimex lectularius and C. Hemipterus. Insects 2, 151–172. doi:10.3390/insects2020151

Benoit, J.B., Lopez-Martinez, G., Patrick, K.R., Phillips, Z.P., Krause, T.B., Denlinger, D.L., 2011. From the Cover: Drinking a hot blood meal elicits a protective heat shock response in mosquitoes. PNAS 108, 8026.

Bernard, C., 2011. Introduction à l'étude de la médecine expérimentale. Le Livre de Poche.

Bigot, L., Shaik, H.A., Bozzolan, F., Party, V., Lucas, P., Debernard, S., Siaussat, D., 2012. Peripheral regulation by ecdysteroids of olfactory responsiveness in male Egyptian cotton leaf worms, Spodoptera littoralis. Insect Biochem. Mol. Biol. 42, 22–31. doi:10.1016/j.ibmb.2011.10.003

Bookstein, F.L., 1997. Morphometric tools for landmark data: geometry and biology. Cambridge University Press.

Bosch, M., Chown, S.L., Scholtz, C.H., 2000. Discontinuous gas exchange and water loss in the keratin beetle Omorgus radula: further evidence against the water conservation hypothesis? Physiol. Entomol. 25, 309–314. doi:10.1111/j.1365-3032.2000.00197.x

Bourtzis, K., Miller, T.A., 2008. Insect Symbiosis. CRC Press.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. doi:10.1016/0003-2697(76)90527-3

Bradley, T.J., Williams, A.E., Rose, M.R., 1999. Physiological Responses to Selection for Desiccation Resistance in Drosophila melanogaster. Am. Zool. 39, 337–345. doi:10.1093/icb/39.2.337

Bradshaw, A.D., 1965. Evolutionary Significance of Phenotypic Plasticity in Plants, in: E.W. Caspari and J.M. Thoday (Ed.), Advances in Genetics. Academic Press, pp. 115–155.

Briegel, H., Waltert, A., Kuhn, R., 2001. Reproductive Physiology of Aedes (Aedimorphus) vexans (Diptera: Culicidae) in Relation to Flight Potential. J. Med. Entomol. 38, 557–565. doi:10.1603/0022-2585-38.4.557

Briegel, H., 2003. Physiological bases of mosquito ecology. J Vector Ecol, 28, 1–11.

Briere, J.F., Pracros, P., Le Roux, A.Y., Pierre, J.S., 1999. A novel rate model of temperature-dependent development for arthropods, Environmental Entomology. vol. 28, no. 1, pp. 22–29.

Browne, J., Tunnacliffe, A., Burnell, A., 2002. Anhydrobiosis: Plant desiccation gene found in a nematode. Nature 416, 38–38. doi:10.1038/416038a

Brunet, P.C., 1963. Tyrosine metabolism in insects. Ann NY Acad Sci, 100,1020–1034.

Bullard, B., Pastore, A., 2011. Regulating the contraction of insect flight muscle. J Muscle Res Cell M, 32, 303–313. doi: 10.1007/s10974-011-9278-1

Bursell, E., 1960. Loss of water by excretion and defaecatlon in the tsetse fly. J. Exp. Biol. 37, 689–697 pp.

C

Campbell, E.M., Ball, A., Hoppler, S., Bowman, A.S., 2008. Invertebrate aquaporins: a review. J. Comp. Physiol. B 178, 935–955. doi:10.1007/s00360-008-0288-2

Campbell, E.M., Burdin, M., Hoppler, S., Bowman, A.S., 2010. Role of an aquaporin in the sheep tick Ixodes ricinus: Assessment as a potential control target. Int. J. Parasitol. 40, 15–23. doi:10.1016/j.ijpara.2009.06.010

Cassone, B.J., Molloy, M.J., Cheng, C., Tan, J.C., Hahn, M.W., Besansky, N.J., 2011. Divergent transcriptional response to thermal stress by Anopheles gambiae larvae carrying alternative arrangements of inversion 2La. Molec Ecol, 20, 2567–2580. doi: 10.1111/j.1365-294X.2011.05114.x

Charlwood, J.D., 1996. Biological variation in Anopheles darlingi root. Mem. Inst. Oswaldo Cruz 91, 391–398. doi:10.1590/S0074-02761996000400001

Charlwood, J.D., Vij, R., Billingsley, P.F., 2000. Dry season refugia of malaria-transmitting mosquitoes in a dry savannah zone of east Africa. Am. J. Trop. Med. Hyg. 62, 726–732.

Charlwood, J.D., Pinto, J., Sousa, C.A., Madsen, H., Ferreira, C., do Rosario, V.E., 2002. The swarming and mating behaviour of Anopheles gambiae s.s. (Diptera: Culicidae) from São Tomé Island. J. Vector Ecol. J. Soc. Vector Ecol. 27, 178–183.

Chouaïbou, M., Etang, J., Brévault, T., Nwane, P., Hinzoumbé, C.K., Mimpfoundi, R., Simard, F., 2008. Dynamics of insecticide resistance in the malaria vector Anopheles gambiae s.l. from an area of extensive cotton cultivation in Northern Cameroon. Trop. Med. Int. Health 13, 476–486. doi:10.1111/j.1365-3156.2008.02025.x

Chown, S.L., 1993. Desiccation Resistance in Six Sub-Antarctic Weevils (Coleoptera: Curculionidae): Humidity as an Abiotic Factor Influencing Assemblage Structure. Funct. Ecol. 7, 318. doi:10.2307/2390211

Chown, S.L., Holter, P., 2000. Discontinuous gas exchange cycles in Aphodius fossor (Scarabaeidae): a test of hypotheses concerning origins and mechanisms. J. Exp. Biol. 203, 397–403.

Chown, S.L., 2002. Respiratory water loss in insects. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 133, 791–804. doi:10.1016/S1095-6433(02)00200-3

Chown, S.L., Nicolson, S., 2004. Insect Physiological Ecology: Mechanisms and Patterns. Oxford University Press.

Chown, S.L., Slabber, S., McGeoch, M.A., Janion, C., Leinaas, H.P., 2007. Phenotypic plasticity mediates climate change responses among invasive and indigenous arthropods. Proc. R. Soc. B Biol. Sci. 274, 2531–2537. doi:10.1098/rspb.2007.0772

Chown, S.L., Hoffmann, A., Kristensen, T., Angilletta, M., Stenseth, N., Pertoldi, C., 2010. Adapting to climate change: a perspective from evolutionary physiology. Clim. Res. 43, 3–15. doi:10.3354/cr00879

Chown, S.L., Sørensen, J.G., Terblanche, J.S., 2011. Water loss in insects: an environmental change perspective. J Insect Physiol, 57, 1070–1084.

Clarke, A., 1993. Seasonal Acclimatization and Latitudinal Compensation in Metabolism: Do They Exist? Funct. Ecol. 7, 139. doi:10.2307/2389880

Claude, J., Bernard, A., Lointier, M., 1981. Observations climatologiques à la station météorologique de jalafanka mare d'0ursi. Office de la recherche scientifique et technique outre-mer.

Clavel, D., Barro, A., Belay, T., Lahmar, R., Maraux, F., 2009. Changements techniques et dynamique d'innovation agricole en Afrique Sahelienne: le cas du Zaï mécanisé au Burkina Faso et de l'introduction d'une cactée en Ethiopie. VertigO - la revue électronique en sciences de l'environnement. doi:10.4000/vertigo.7442

Clements, A.N., 2012. The Biology of Mosquitoes, Volume 3 Transmission of Viruses and Interactions with Bacteria. CABI.

Cloudsley-Thompson, J., 2001. Thermal and water relations of desert beetles. Naturwissenschaften 88, 447–460. doi:10.1007/s001140100256

Coast, G.M., Orchard, I., Phillips, J.E., Schooley, D.A., 2002. Insect diuretic and antidiuretic hormones, in: Advances in Insect Physiology. Academic Press, pp. 279–409.

Coetzee, M., Craig, M., le Sueur, D., 2000. Distribution of African Malaria Mosquitoes Belonging to the Anopheles gambiae Complex. Parasitol. Today 16, 74–77. doi:10.1016/S0169-4758(99)01563-X

Coetzee, M., Hunt, R.H., Wilkerson, R., della Torre, A., Coulibaly, M.B., Besansky, N.J., 2013. Anopheles coluzzii and Anopheles amharicus, new members of the Anopheles gambiae complex. Zootaxa, 3619(2), 246–274.

Colinet, H., Renault, D., Charoy-Guével, B., Com, E., 2012. Metabolic and proteomic profiling of diapause in the aphid parasitoid Praon volucre. PLoS ONE, 7, e32606. doi: 10.1371/journal.pone.0032606

Colinet, H., Overgaard, J., Com, E., Sørensen, J.G., 2013. Proteomic profiling of thermal acclimation in Drosophila melanogaster. Insect Biochem Molec Biol, 43, 352–365. doi: 10.1016/j.ibmb.2013.01.006

Colinet, H., Renault, D., 2014. Dietary live yeast alters metabolic profiles, protein biosynthesis and thermal stress tolerance of Drosophila melanogaster. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 170, 6–14. doi:10.1016/j.cbpa.2014.01.004

Collyer, M.L., Adams, D.C., 2007. Analysis of two-state multivariate phenotypic change in ecological studies. Ecology. 88, 683–692. doi:10.2307/27651151

Coluzzi, M., Sabatini, A., Petrarca, V., Deco, M.A.D., 1979. Chromosomal differentiation and adaptation to human environments in the Anopheles gambiae complex. Trans. R. Soc. Trop. Med. Hyg. 73, 483–497. doi:10.1016/0035-9203(79)90036-1

Coluzzi, M., Sabatini, A., Torre, A. della, Deco, M.A.D., Petrarca, V., 2002. A Polytene chromosome analysis of the Anopheles gambiae species complex. Science 298, 1415–1418. doi:10.1126/science.1077769

Cooper, P.D., 1985. Seasonal Changes in Water Budgets in Two Free-Ranging Tenebrionid Beetles, Eleodes armata and Cryptoglossa verrucosa. Physiol. Zool. 58, 458-472

Costantini, C., Ayala, D., Guelbeogo, W.M., Pombi, M., Some, C.Y., Bassole, I.H., Ose, K., Fotsing, J.-M., Sagnon, N., Fontenille, D., Besansky, N.J., Simard, F., 2009. Living at the edge: biogeographic patterns of habitat segregation conform to speciation by niche expansion in Anopheles gambiae. BMC Ecology. 9, 16. doi:10.1186/1472-6785-9-16

Couret, J., Dotson, E., Benedict, M.Q., 2014. Temperature, larval diet, and density effects on development rate and survival of Aedes aegypti (Diptera: Culicidae). PLoS ONE. 9, e87468. doi:10.1371/journal.pone.0087468

Craig, M.H., Snow, R.W., le Sueur, D., 1999. A Climate-based Distribution Model of Malaria Transmission in Sub-Saharan Africa. Parasitol. Today 15, 105–111. doi:10.1016/S0169-4758(99)01396-4

Crawley, M.J., 2007. The R Book. John Wiley & Sons.

Crowe, J., 1992. Anhydrobiosis. Annu. Rev. Physiol. 54, 579–599. doi:10.1146/annurev.physiol.54.1.579

Cushman, J.C., Bohnert, H.J., 2000. Genomic approaches to plant stress tolerance. Current Opinion in Plant Biology 3, 117–124. doi:10.1016/S1369-5266(99)00052-7

Czarnoleski, M., Cooper, B.S., Kierat, J., Angilletta, M.J., 2013. Flies developed small bodies and small cells in warm and in thermally fluctuating environments. J. Exp. Biol. 216, 2896–2901. doi:10.1242/jeb.083535

D

Dabiré, K.R., Diabaté, A., Namontougou, M., Djogbenou, L., Kengne, P., Simard, F., Bass, C., Baldet, T., 2009. Distribution of insensitive acetylcholinesterase (ace-1R) in Anopheles gambiae s.l. populations from Burkina Faso (West Africa). Trop. Med. Int. Health 14, 396–403. doi:10.1111/j.1365-3156.2009.02243.x

Dabiré, K.R., Baldet, T., Diabaté, A., Dia, I., Costantini, C., Cohuet, A., Guiguemdé, T.R., Fontenille, D., 2007. Anopheles funestus (Diptera: Culicidae) in a Humid Savannah Area of Western Burkina Faso: Bionomics, Insecticide Resistance Status, and Role in Malaria Transmission. J. Med. Entomol. 44, 990–997. doi:10.1603/0022-2585(2007)44[990:AFDCIA]2.0.CO;2

Damos, P., Savopoulou-Soultani, M., 2012. Temperature-driven models for insect development and vital thermal requirements. Psyche: A Journal of Entomology. 2012, 1–13. doi:10.1155/2012/123405

Danks, H.V., 1987. Insect Dormancy: An Ecological Perspective (Biological Survey of Canada Monograph Series), H. , No 1.

Danks, H.V., 2000. Dehydration in dormant insects. J. Insect Physiol. 46, 837–852.

Davidson, A.M., Jennions, M., Nicotra, A.B., 2011. Do invasive species show higher phenotypic plasticity than native species and, if so, is it adaptive? A meta-analysis. Ecol. Lett. 14, 419–431. doi:10.1111/j.1461-0248.2011.01596.x

Davies, S.-A., Cabrero, P., Povsic, M., Johnston, N.R., Terhzaz, S., Dow, J.A.T., 2013. Signaling by Drosophila capa neuropeptides. Gen. Comp. Endocrinol., 26th Conference of European Comparative Endocrinologists (CECE) with Special Section on Fish Model system edited by Oliana Carnevali 26th Conference of European Comparative Endocrinologists 188, 60–66. doi:10.1016/j.ygcen.2013.03.012

Davis, A.L.V., Chown, S.L., McGeoch, M.A., Scholtz, C.H., 2000. A comparative analysis of metabolic rate in six Scarabaeus species (Coleoptera: Scarabaeidae) from southern Africa: further caveats when inferring adaptation. J. Insect Physiol. 46, 553–562. doi:10.1016/S0022-1910(99)00141-9

Debat, V., Cornette, R., Korol, A ., Nevo, E., Soulet, D., David, J.R., 2008. Multidimensional analysis of Drosophila wing in Evolution canyon. Journal of Genetics. 87 : 407–419.

Debat, V., Bloyer, S., Faradji, F., Gidaszewski, N., Navarro, N., Orozco-terWengel, P., Ribeiro, V., Schlötterer, C., Deutsch, J.S., Peronnet, F., 2011. Developmental Stability: A Major Role for Cyclin G in Drosophila melanogaster. PLoS Genet. 7, e1002314. doi:10.1371/journal.pgen.1002314

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E., Boulétreau, M., 2001. Removing symbiotic Wolbachia bacteria specifically inhibits oogenesis in a parasitic wasp. Proc. Natl. Acad. Sci. 98, 6247–6252. doi:10.1073/pnas.101304298

Denlinger, D.L., Yocum, G.D., 1998. Physiology of heat sensitivity. Temp. Sensit. Insects Appl. Integr. Pest Manag. 7–53.

Denlinger, D.L., 2002. Regulation of Diapause. Annu. Rev. Entomol. 47, 93–122. doi:10.1146/annurev.ento.47.091201.145137

Denlinger, D.L., Armbruster, P.A., 2014. Mosquito Diapause. Annu. Rev. Entomol. 59, 131025101253002. doi:10.1146/annurev-ento-011613-162023

Diabate, A., Baldet, T., Chandre, F., Akoobeto, M., Guiguemde, T.R., Darriet, F., Brengues, C., Guillet, P., Hemingway, J., Small, G.J., 2002. The role of agricultural use of insecticides in resistance to pyrethroids in Anopheles gambiae s.l. in Burkina Faso. Am. J. Trop. Med. Hyg. 67, 617–622.

Diabate, A., Brengues, C., Baldet, T., Dabiré, K.R., Hougard, J.M., Akogbeto, M., Kengne, P., Simard, F., Guillet, P., Hemingway, J., Chandre, F., 2004. The spread of the Leu-Phe kdr mutation through Anopheles gambiae complex in Burkina Faso: genetic introgression and de novo phenomena. Trop. Med. Int. Health 9, 1267–1273. doi:10.1111/j.1365-3156.2004.01336.x

Dillon, M.E., Frazier, M.R., Dudley, R., 2006. Into thin air: physiology and evolution of alpine insects. Integrative and Comparative Biology. 46, 49–61.

Djawdan, M., Rose, M.R., Bradley, T.J., 1997. Does selection for stress resistance lower metabolic rate? Ecology 78, 828–837. doi:10.1890/0012-9658(1997)078[0828:DSFSRL]2.0.CO;2

Doggett, S.L., Geary, M.J., Russell, R.C., 2004. The Resurgence of Bed Bugs in Australia: With Notes on Their Ecology and Control.

Drake, V.A., 1995. Insect Migration: Tracking Resources Through Space and Time. Cambridge University Press.

Dujardin, J.-P., Slice, D., 2007. Geometric morphometrics: contributions to medical entomology. In: Tibayrenc M, editor. Encyclopedia of infectious diseases. Modern methodologies. Vol. 25. Wiley & Sons; Hoboken, NJ: 2007. pp. 435–447

Dujardin, J.-P., 2008. Morphometrics applied to medical entomology. Infection, Genetics and Evolution. 8: 875-890

Dujardin, J.-P., 2011. Modern morphometrics of medically important insects. In: Tibayrenc, M., (Ed.), Genetics and evolution of infectious diseases, Elsevier, pp. 473–501 (Chapter 16) ISBN: 978-0-12-384890-1, p. 749.

E

Edney, E.B., 1977. Water balance in land arthropods. 282pp.

Elnitsky, M.A., Hayward, S.A.L., Rinehart, J.P., Denlinger, D.L., Lee, R.E., 2008. Cryoprotective dehydration and the resistance to inoculative freezing in the Antarctic midge, Belgica antarctica. J Exp Biol, 211, 524–530.

F

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and Ecological Physiology. Annu. Rev. Physiol. 61, 243–282. doi:10.1146/annurev.physiol.61.1.243

Fernandes, L., Briegel, H., 2005. Reproductive physiology of Anopheles gambiae and Anopheles atroparvus. J. Vector Ecol. 30, 11.

Fiil, D.A., 1976. Oogenesis in the malaria mosquito Anopheles gambiae. Cell Tissue Res. 167, 23–35. doi:10.1007/BF00220157

Folk, D.G., Bradley, T.J., 2004. The evolution of recovery from desiccation stress in laboratory-selected populations of Drosophila melanogaster. J. Exp. Biol. 207, 2671–2678. doi:10.1242/jeb.01048

Fontenille, D., Lochouarn, L., Diagne, N., Sokhna, C., Lemasson, J.J., Diatta, M., Konate, L., Faye, F., Rogier, C., Trape, J.F., 1997. High annual and seasonal variations in malaria transmission by anophelines and vector species composition in Dielmo, a holoendemic area in Senegal. Am J Trop Med Hyg, 56, 247–253.

Fontenille, D., Cohuet, A., Awono-Ambene, P., Antonio-Nkondjio, C., Wondji, C., Kengne, P., Dia, I., Boccolini, D., Duchemin, J.B., Rajanonarivelo, V., Dabire, R., Adja-Akre, M., Ceainu, C., Le Goff, G., Simard, F., 2003. Systématique et biologie des Anopheles vecteurs de Plasmodium en Afrique, données récentes. Médecine Trop. 63, 247–253.

Fontenille, D., Simard, F., 2004. Unravelling complexities in human malaria transmission dynamics in Africa through a comprehensive knowledge of vector populations. Comp. Immunol. Microbiol. Infect. Dis. 27, 357–375.

Foray, V., Pelisson, P.-F., Bel-Venner, M.-C., Desouhant, E., Venner, S., Menu, F., Giron, D., Rey, B., 2012. A handbook for uncovering the complete energetic budget in insects: the van Handel's method (1985) revisited. Physiol. Entomol. 37, 295–302. doi:10.1111/j.1365-3032.2012.00831.x

Foray, V., Desouhant, E., Voituron, Y., Larvor, V., Renault, D., Colinet, H., Gibert, P., 2013. Does cold tolerance plasticity correlate with the thermal environment and metabolic profiles of a parasitoid wasp? Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164, 77–83. doi:10.1016/j.cbpa.2012.10.018

Foster, W.A., 1995. Mosquito Sugar Feeding and Reproductive Energetics. Annu. Rev. Entomol. 40, 443–474. doi:10.1146/annurev.en.40.010195.002303

Foster, W.A., Takken, W., 2004. Nectar-related vs. human-related volatiles: behavioural response and choice by female and male Anopheles gambiae (Diptera: Culicidae) between emergence and first feeding. Bull. Entomol. Res. 94, 145–157.

Fouet, C., Gray, E., Besansky, N.J., Costantini, C., 2012. Adaptation to aridity in the malaria mosquito Anopheles gambiae: chromosomal inversion polymorphism and body size influence resistance to desiccation. PLoS ONE, 7 (4), e34841. doi:10.1371/journal.pone.0034841.

Freeman, S., 2007. Evolutionary Analysis 4th edition by Freeman, Scott, Herron, Jon C. Benjamin Cumings,2007. 4th edition.

Fry, S.N., Sayaman, R., Dickinson, M.H., 2003. The aerodynamics of free-flight maneuvers in Drosophila. Science. 300, 495–498. doi:10.1126/science.1081944

G

Gäde, G., 2004. Regulation of Intermediary Metabolism and Water Balance of Insects by Neuropeptides. Annu. Rev. Entomol. 49, 93–113. doi:10.1146/annurev.ento.49.061802.123354

Gal, T.Z., Solomon, A., Glazer, I., Koltai, H., 2001. Alterations in the Levels of Glycogen and Glycogen Synthase Transcripts During Desiccation in the Insect-Killing Nematode Steinernema Feltiae IS-6. J. Parasitol. 87, 725–732. doi:10.1645/0022-3395(2001)087[0725:AITLOG]2.0.CO;2

Gatz, A.J., 1979. Community organization in fishes as indicated by morphological features. Evolution. 60, 711-718.

Gething, M.-J., Sambrook, J., 1992. Protein folding in the cell. Nature 355, 33–45. doi:10.1038/355033a0

Ghalambor, C.K., McKAY, J.K., Carroll, S.P., Reznick, D.N., 2007. Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. Funct. Ecol. 21, 394–407. doi:10.1111/j.1365-2435.2007.01283.x

Gibbs, A.G., Chippindale, A.K., Rose, M.R., 1997. Physiological mechanisms of evolved desiccation resistance in Drosophila melanogaster. J. Exp. Biol. 200, 1821–1832.

Gibbs, A.G., Louie, A.K., Ayala, J.A., 1998. Effects of temperature on cuticular lipids and water balance in a desert Drosophila: is thermal acclimation beneficial? J. Exp. Biol. 201, 71–80.

Gibbs, A.G., 2002. Water balance in desert Drosophila: lessons from non-charismatic microfauna. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 133, 781–789. doi:10.1016/S1095-6433(02)00208-8

Gibbs, A.G., Fukuzato, F., Matzkin, L.M., 2003. Evolution of water conservation mechanisms in Drosophila. J. Exp Biol, 206, 1183–1192. doi: 10.1242/jeb.00233

Gibbs, A.G., Rajpurohit, S., 2010. Cuticular lipids and water balance. Insect Hydrocarb. 100–120.

Gilchrist, A.S., Azevedo, R.B.R., Partridge, L., O'higgins, P., 2000. Adaptation and constraint in the evolution of Drosophila melanogaster wing shape. Evolution & Development. 2, 114–124. doi:10.1046/j.1525-142x.2000.00041.x

Gilles, R., 2006. Physiologie animale. De Boeck Supérieur.

Gillies, M.T., De Meillon, B., 1968. The Anophelinae of Africa south of the Sahara (Ethiopian Zoogeographical Region). 343 pp.

Gimonneau, G., Pombi, M., Choisy, M., Morand, S., Dabiré, R.K., Simard, F., 2012a. Larval habitat segregation between the molecular forms of the mosquito Anopheles gambiae in a rice field area of Burkina Faso, West Africa. Medical and Veterinary Entomology. 26, 9–17. doi:10.1111/j.1365-2915.2011.00957.x

Gimonneau, G., Bouyer, J., Morand, S., Besansky, N.J., Diabate, A., Simard, F., 2010b. A behavioral mechanism underlying ecological divergence in the malaria mosquito Anopheles gambiae. Behav. Ecol. 21, 1087–1092. doi:10.1093/beheco/arq114

Gimonneau, G., Pombi, M., Dabiré, R.K., Diabaté, A., Morand, S., Simard, F., 2012b. Behavioural responses of Anopheles gambiae sensu stricto M and S molecular form larvae to an aquatic predator in Burkina Faso. Parasit Vectors 5, 65.

Glass, E.V., Yoder, J.A., Needham, G.R., 1998. Clustering reduces water loss by adult American house dust mites Dermatophagoides farinae (Acari : Pyroglyphidae). Exp. Appl. Acarol. 22, 31–37. doi:10.1023/A:1006081323887

Goldspink, G., Scutt, A., Loughna, P.T., Wells, D.J., Jaenicke, T., Gerlach, G.F., 1992. Gene expression in skeletal muscle in response to stretch and force generation. Am J Physiology, 262, R356–R363.

Goltsev, Y., Rezende, G.L., Vranizan, K., Lanzaro, G., Valle, D., Levine, M., 2009. Developmental and evolutionary basis for drought tolerance of the Anopheles gambiae embryo. Dev Biol, 330, 462–470. doi: 10.1016/j.ydbio.2009.02.038

Goyal, K., Pinelli, C., Maslen, S.L., Rastogi, R.K., Stephens, E., Tunnacliffe, A., 2005. Dehydration-regulated processing of late embryogenesis abundant protein in a desiccation-tolerant nematode. FEBS Letters 579, 4093–4098. doi:10.1016/j.febslet.2005.06.036

Gray, E.M., Bradley, T.J., 2006. Evidence from mosquitoes suggests that cyclic gas exchange and discontinuous gas exchange are two manifestations of a single respiratory pattern. J. Exp. Biol. 209, 1603–1611.

Gray, E.M., Rocca, K.A., Costantini, C., Besansky, N.J., 2009. Inversion 2La is associated with enhanced desiccation resistance in Anopheles gambiae. Malar. J. 8, 215.

Grimstone, A.V., Mullinger, A.M., Ramsay, J.A., 1968. Further Studies on the Rectal Complex of the Mealworm Tenebrio molitor, L. (Coleoptera, Tenebrionidae). Philos. Trans. R. Soc. Lond. B. Biol. Sci. 253, 343–382. doi:10.1098/rstb.1968.0004

H

Haas, H., Tolley, K.A., 1998. Geographic variation of wing morphology in three Eurasian populations of the fruit fly, Drosophila lummei. Journal of Zoology (London). 245, 197-203.

Hadley, N.F., 1994. Water relations of terrestrial arthropods. Academic Press.

Hahn, D.A., Denlinger, D.L., 2007. Meeting the energetic demands of insect diapause: Nutrient storage and utilization. J. Insect Physiol. 53, 760–773. doi:10.1016/j.jinsphys.2007.03.018

Hahn, D.A., Denlinger, D.L., 2011. Energetics of insect diapause. Annu. Rev. Entomol. 56, 103–121.

Hamilton, W.D., 1964. The genetical evolution of social behaviour. I. J. Theor. Biol. 7, 1–16. doi:10.1016/0022-5193(64)90038-4

Harbach, R. e., 2004. The classification of genus Anopheles (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bull. Entomol. Res. 94, 537–553. doi:10.1079/BER2004321

Hardie, D.G., 2008. AMPK: a key regulator of energy balance in the single cell and the whole organism. Int. J. Obes. 2005 32 Suppl 4, S7–12. doi:10.1038/ijo.2008.116

Hargreaves, K., Koekemoer, L.L., Brooke, B.D., Hunt, R.H., Mthembu, J., Coetzee, M., 2000. Anopheles funestus resistant to pyrethroid insecticides in South Africa. Med. Vet. Entomol. 14, 181–189. doi:10.1046/j.1365-2915.2000.00234.x

Harris, C., Lambrechts, L., Rousset, F., Abate, L., Nsango, S.E., Fontenille, D., Morlais, I., Cohuet, A., 2010. Polymorphisms in Anopheles gambiae immune genes associated with natural resistance to Plasmodium falciparum. PLoS Pathogens. 6, e1001112. doi:10.1371/journal.ppat.1001112

Hayward, S.A.L., Rinehart, J.P., Denlinger, D.L., 2004. Desiccation and rehydration elicit distinct heat shock protein transcript responses in flesh fly pupae. J. Exp. Biol. 207, 963–971. doi:10.1242/jeb.00842

Hayward, S.A., 2014. Application of functional "Omics" in environmental stress physiology: insights, limitations, and future challenges. Curr. Opin. Insect Sci. doi:10.1016/j.cois.2014.08.005

Hepburn, H.R., Youthed, C., Illgner, P., Radloff, S.E., Brown, R.E., 1998. Production of aerodynamic power in mountain honeybees (Apis mellifera). Naturwissenschaften. 85, 389–390. doi:10.1007/s001140050520

Hoback, W.W., Stanley, D.W., 2001. Insects in hypoxia. J. Insect Physiol. 47, 533–542. doi:10.1016/S0022-1910(00)00153-0

Hoekstra, F.A., Golovina, E.A., Buitink, J., 2001. Mechanisms of plant desiccation tolerance. Trends Plant Sci. 6, 431–438. doi:10.1016/S1360-1385(01)02052-0

Hoffman, A.A., Parsons, P.A., 1991. Evolutionary Genetics and Environmental Stress. Oxford University Press. paper ix, 284pp.

Hoffman, A.A., Shirrifs, J., 2002. Geographic variation for wing shape in Drosophila serrata. Evolution. 56, 1068-1073.

Hoffmann, A.A., Harshman, L.G., 1999. Desiccation and starvation resistance in Drosophila: patterns of variation at the species, population and intrapopulation levels. Heredity, 83, 637–643. doi:10.1046/j.1365-2540.1999.00649.x

Hoffmann, A.A., Parsons, P.A., 1989. An integrated approach to environmental stress tolerance and life-history variation: desiccation tolerance in Drosophila. Biol. J. Linn. Soc. 37, 117–136. doi:10.1111/j.1095-8312.1989.tb02098.x

Hoffmann, A.A., Parsons, P.A., 1993. Direct and correlated responses to selection for desiccation resistance: a comparison of Drosophila melanogaster and D. simulans. J. Evol. Biol. 6, 643–657. doi:10.1046/j.1420-9101.1993.6050643.x

Holmes, K.C., 2011. Steric blocking mechanism explains stretch activation in insect flight muscle. Proc Natl Acad Sci USA, 108, 7–8. doi:10.1073/pnas.1017429108

Holmstrup, M., Sjursen, H., Ravn, H., Bayley, M., 2001. Dehydration tolerance and water vapour absorption in two species of soil-dwelling Collembola by accumulation of sugars and polyols. Funct. Ecol. 15, 647–653. doi:10.1046/j.0269-8463.2001.00565.x

Holstein, M.H., 1954. Biology of Anopheles gambiae: research in French West Africa. World Health Organization.

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.C., Wides, R., Salzberg, S.L., Loftus, B., Yandell, M., Majoros, W.H., Rusch, D.B., Lai, Z., Kraft, C.L., Abril, J.F., Anthouard, V., Arensburger, P., Atkinson, P.W., Baden, H., Berardinis, V. de, Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chatuverdi, K., Christophides, G.K., Chrystal, M.A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C.A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M.E., Hladun, S.L., Hogan, J.R., Hong, Y.S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J.-J., Lobo, N.F., Lopez, J.R., Malek, J.A., McIntosh, T.C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S.D., O'Brochta, D.A., Pfannkoch, C., Qi, R., Regier, M.A., Remington, K., Shao, H., Sharakhova, M.V., Sitter, C.D., Shetty, J., Smith, T.J., Strong, R., Sun, J., Thomasova, D., Ton, L.Q., Topalis, P., Tu, Z., Unger, M.F., Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K.J., Wortman, J.R., Wu, M., Yao, A., Zdobnov, E.M., Zhang, H., Zhao, Q., Zhao, S., Zhu, S.C., Zhimulev, I., Coluzzi, M., Torre, A. della, Roth, C.W., Louis, C., Kalush, F., Mural, R.J., Myers, E.W., Adams, M.D., Smith, H.O., Broder, S., Gardner, M.J., Fraser, C.M., Birney, E., Bork, P., Brey, P.T., Venter, J.C., Weissenbach, J., Kafatos, F.C., Collins, F.H., Hoffman, S.L., 2002. The Genome Sequence of the Malaria Mosquito Anopheles gambiae. Science 298, 129–149. doi:10.1126/science.1076181

Horman, S., Hussain, N., Dilworth, S.M., Storey, K.B., Rider, M.H., 2005. Evaluation of the role of AMP-activated protein kinase and its downstream targets in mammalian hibernation. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 142, 374–382. doi:10.1016/j.cbpb.2005.08.010

Huestis, D.L., Lehmann, T., 2014. Ecophysiology of Anopheles gambiae s.l.: persistence in the Sahel. Infection, Genetics and Evolution. doi:10.1016/j.meegid.2014.05.027

Huestis, D.L., Traoré, A.I., Adamou, A., Kassogué, Y., Diallo, M., Timbiné, S., Dao, A., Lehmann, T., 2011. Variation in metabolic rate of Anopheles gambiae and A. arabiensis in a Sahelian village. J. Exp. Biol. 214, 2345–2353.

Huestis, D.L., Yaro, A.S., Traoré, A.I., Dieter, K.L., Nwagbara, J.I., Bowie, A.C., Adamou, A.Y., Diallo, M., Timbiné, S., Dao, A., Lehmann, T., 2012. Seasonal variation in metabolic rate, flight activity and body size of Anopheles gambiae in the Sahel. J Exp Biol, 215, 2013–2021. doi:10.1242/jeb.069468.

Huey, R.B., Gilchrist, G.W., Carlson, M.L., Berrigan, D., Serra, L., 2000. Rapid evolution of a geographic cline in size in an introduced fly. Science. 287, 308–309. doi:10.1126/science.287.5451.308

I

Iconomidou, V.A., Willis, J.H., Hamodrakas, S.J., 2005. Unique features of the structural model of "hard" cuticle proteins: implications for chitin–protein interactions and cross-linking in cuticle. Insect Biochem Molec Biol, 35, 553–560.

Im, A.R., Park, Y., Sim, J.S., Zhang, Z., Liu, Z., Linhardt, R.J., Kim, Y.S., 2010. Glycosaminoglycans from earthworms (Eisenia andrei). Glycoconjugate J, 27, 249–257.

Isabel, G., Martin, J.-R., Chidami, S., Veenstra, J.A., Rosay, P., 2005. AKH-producing neuroendocrine cell ablation decreases trehalose and induces behavioral changes in Drosophila. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 288, R531–R538. doi:10.1152/ajpregu.00158.2004

Izumi, Y., Sonoda, S., Yoshida, H., Danks, H.V., Tsumuki, H., 2006. Role of membrane transport of water and glycerol in the freeze tolerance of the rice stem borer, Chilo suppressalis Walker (Lepidoptera: Pyralidae). J. Insect Physiol. 52, 215–220. doi:10.1016/j.jinsphys.2005.11.001

J

Jensen, J.L.W.V., 1906. Sur les fonctions convexes et les inégalités entre les valeurs moyennes. Acta Math. 30, 175–193. doi:10.1007/BF02418571

Jirakanjanakit, N., Dujardin, J.-P., 2005. Discrimination of Aedes aegypti (Diptera: Culicidae) laboratory lines based on wing geometry. The Southeast Asian Journal of Tropical Medicine and Public Health. 36(4):858-61.

Jirakanjanakit, N., Leemingsawat, S., Dujardin, J.-P., 2008. The geometry of the wing of Aedes (Stegomyia) aegypti in isofemale lines through successive generations. Infection, Genetics and Evolution. 8, 414–421. doi:10.1016/j.meegid.2007.05.004

Jirakanjanakit, N., Leemingsawat, S., Thongrungkiat, S., Apiwathnasorn, C., Singhaniyom, S., Bellec, C., Dujardin, J.-P., 2007. Influence of larval density or food variation on the geometry of the wing of Aedes (Stegomyia) aegypti. Tropical Medicine and International Health. 12, 1354–1360. doi:10.1111/j.1365-3156.2007.01919.x

Johnson, R.A., Gibbs, A.G., 2004. Effect of mating stage on water balance, cuticular hydrocarbons and metabolism in the desert harvester ant, Pogonomyrmex barbatus. J. Insect Physiol. 50, 943–953. doi:10.1016/j.jinsphys.2004.07.006

K

Kaersgaard, C.W., Holmstrup, M., Malte, H., Bayley, M., 2004. The importance of cuticular permeability, osmolyte production and body size for the desiccation resistance of nine species of Collembola. J. Insect Physiol. 50, 5–15. doi:10.1016/j.jinsphys.2003.09.003

Kambule, I.N., Hanrahan, S.A., Duncan, F.D., 2011. Metabolic rate in diapause and nondiapause brown locust eggs correlated with embryonic development. Physiol. Entomol. 36, 299–308. doi:10.1111/j.1365-3032.2011.00792.x

Karouzou, M.V., Spyropoulos, Y., Iconomidou, V.A., Cornman, R.S., Hamodrakas, S.J., Willis, J.H., 2007. Drosophila cuticular proteins with the R&R Consensus: Annotation and classification with a new tool for discriminating RR-1 and RR-2 sequences. Insect Biochem. Mol. Biol. 37, 754–760.

Kaufmann, C., Brown, M.R., 2006. Adipokinetic hormones in the African malaria mosquito, Anopheles gambiae: Identification and expression of genes for two peptides and a putative receptor. Insect Biochem. Mol. Biol. 36, 466–481. doi:10.1016/j.ibmb.2006.03.009

Kaufmann, C., Brown, M.R., 2008. Regulation of carbohydrate metabolism and flight performance by a hypertrehalosaemic hormone in the mosquito Anopheles gambiae. J. Insect Physiol. 54, 367–377. doi:10.1016/j.jinsphys.2007.10.007

Kawecki, T.J., Ebert, D., 2004. Conceptual issues in local adaptation. Ecol. Lett. 7, 1225–1241. doi:10.1111/j.1461-0248.2004.00684.x

Kikawada, T., Saito, A., Kanamori, Y., Fujita, M., Śnigórska, K., Watanabe, M., Okuda, T., 2008. Dehydration-inducible changes in expression of two aquaporins in the sleeping chironomid, Polypedilum vanderplanki. Biochim. Biophys. Acta BBA - Biomembr. 1778, 514–520. doi:10.1016/j.bbamem.2007.11.009

Kingsolver, J.G., Ragland, G.J., Diamond, S.E., 2009. Evolution in a constant environment: thermal fluctuations and thermal sensitivity of laboratory and field populations of Manduca sexta. Evolution. 63, 537-41

Kjærsgaard, A., Pertoldi, C., Loeschcke, V., Blanckenhorn, W.U., 2013. The effect of fluctuating temperatures during development on fitness-related traits of Scatophaga stercoraria (Diptera: Scathophagidae). Environmental Entomology. 42,1069-1078

Klingenberg, C. P., Marugán-Lobón, J., 2013. Evolutionary covariation in geometric morphometric data: analyzing integration, modularity and allometry in a phylogenetic context. Systematic Biology. 62,591–610.

Klingenberg, C.P., McIntyre, G.S., 1998. Geometric morphometrics of developmental instability: analyzing patterns of fluctuating asymmetry with procrustes methods. Evolution. 52, 1363. doi:10.2307/2411306

Kodrík, D., Socha, R., Syrová, Z., 2003. Developmental and diel changes of adipokinetic hormone in CNS and haemolymph of the flightless wing-polymorphic bug, Pyrrhocoris apterus (L.). J. Insect Physiol. 49, 53–61. doi:10.1016/S0022-1910(02)00245-7

Koella, J.C., Lyimo, E.O., 1996. Variability in the relationship between weight and wing length of Anopheles gambiae (Diptera: Culicidae). Journal of Medical Entomology. 33, 261–264.

Koenraadt, C.J.M., Paaijmans, K.P., Githeko, A.K., Knols, B.G.J., Takken, W., 2003. Egg hatching, larval movement and larval survival of the malaria vector Anopheles gambiae in desiccating habitats. Malaria J, 2, 20–29. doi:10.1186/1475-2875-2-20

Koenraadt, C.J.M., Takken, W., 2003. Cannibalism and predation among larvae of the Anopheles gambiae complex. Med. Vet. Entomol. 17, 61–66.

König, M., Agrawal, O.P., Schenkel, H., Scheller, K., 1986. Incorporation of calliphorin into the cuticle of the developing blowfly, Calliphora vicina. Roux's Arch Dev Biol, 195, 296–301.

Koštál, V., 2006. Eco-physiological phases of insect diapause. J. Insect Physiol. 52, 113–127. doi:10.1016/j.jinsphys.2005.09.008

Koštál, V., Sula, J., Simek, P., 1998. Physiology of drought tolerance and cold hardiness of the Mediterranean tiger moth Cymbalophora pudica during summer diapause. J. Insect Physiol. 44, 165–173. doi:10.1016/S0022-1910(97)00047-4

Koštál, V., Tollarová, M., Šula, J., 2004. Adjustments of the enzymatic complement for polyol biosynthesis and accumulation in diapausing cold-acclimated adults of Pyrrhocoris apterus. J. Insect Physiol. 50, 303–313. doi:10.1016/j.jinsphys.2004.01.006

Kruger, N.J., von Schaewen, A., 2003. The oxidative pentose phosphate pathway: structure and organisation. Curr. Opin. Plant Biol. 6, 236–246. doi:10.1016/S1369-5266(03)00039-6

Kültz, D., 2005. Molecular and Evolutionary Basis of the Cellular Stress Response. Annu. Rev. Physiol. 67, 225–257. doi:10.1146/annurev.physiol.67.040403.103635

L

Laparie, M., Bical, R., Larvor, V., Vernon, P., Frenot, Y., Renault, D., 2012. Habitat phenotyping of two sub-Antarctic flies by metabolic fingerprinting: Evidence for a species outside its home? Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 162, 406–412. doi:10.1016/j.cbpa.2012.04.022

Lee, G., Park, J.H., 2004. Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in Drosophila melanogaster. Genetics 167, 311–323. doi:10.1534/genetics.167.1.311

Lee, Y., Marsden, C.D., Norris, L.C., Collier, T.C., Main, B.J., Fofana, A., Cornel, A.J., Lanzaro, G.C., 2013. Spatiotemporal dynamics of gene flow and hybrid fitness between the M and S forms of the malaria mosquito, Anopheles gambiae. Proc. Natl. Acad. Sci. 110, 19854–19859.

Lee, Y., Meneses, C.R., Fofana, A., Lanzaro, G.C., 2009. Desiccation resistance among subpopulations of Anopheles gambiae s.s. From Selinkenyi, Mali. J. Med. Entomol. 46, 316–320. doi:10.1603/033.046.0216

Lehmann, T., Dao, A., Adamou, A., Kassogue, Y., Diallo, M., Sékou, T., Coscaron-Arias, C., 2010. Aestivation of the African malaria mosquito, Anopheles gambiae in the Sahel. The American journal of tropical medicine and hygiene. 83, 601–606.

Lehmann, T., Dao, A., Yaro, A.S., Diallo, M., Timbiné, S., Huestis, D.L., Adamou, A., Kassogué, Y., Traoré, A.I., 2014. Seasonal Variation in Spatial Distributions of Anopheles gambiae in a Sahelian Village: Evidence for Aestivation. J. Med. Entomol. 51, 27–38. doi:10.1603/ME13094

Lehmann, T., Diabate, A., 2008. The molecular forms of Anopheles gambiae: A phenotypic perspective. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 8, 737–746. doi:10.1016/j.meegid.2008.06.003

Lemasson, J.J., Fontenille, D., Lochouarn, L., Dia, I., Simard, F., Ba, K., Diop, A., Diatta, M., Molez, J.F., 1997. Comparison of behavior and vector efficiency of Anopheles gambiae and An. arabiensis (Diptera:Culicidae) in Barkedji, a Sahelian area of Senegal. J Med Entomol, 34, 396–403.

Levins, R., 1968. Evolution in Changing Environments: Some Theoretical Explorations. Princeton University Press.

Levis, N.A., Yi, S.-X., Lee, R.E., 2012. Mild desiccation rapidly increases freeze tolerance of the goldenrod gall fly, Eurosta solidaginis: evidence for drought-induced rapid cold-hardening. J. Exp. Biol. 215, 3768–3773. doi:10.1242/jeb.076885

Li, A., Denlinger, D.L., 2009. Pupal Cuticle Protein is Abundant During Early Adult Diapause in the Mosquito Culex pipiens. J. Med. Entomol. 46, 1382–1386. doi:10.1603/033.046.0618

Lighton, J., 1992. Direct Measurement of Mass Loss During Discontinuous Ventilation in Two Species of Ants. J. Exp. Biol. 173, 289–293.

Lighton, J.R.B., Garrigan, D.A., Duncan, F.D., Johnson, R.A., 1993. Spiracular Control of Respiratory Water Loss in Female Alates of the Harvester Ant Pogonomyrmex Rugosus. J. Exp. Biol. 179, 233–244.

Losos, J.B., Sinerov, B., 1989. The effect of morphology and perch size on sprint performance in Anolis lizards. Journal of Experimental Biology. 145: 23-30.

Lounibos, L.P., Suárez, S., Menéndez, Z., Nishimura, N., Escher, R.L., O'Connell, S.M., Rey, J.R., 2002. Does temperature affect the outcome of larval competition between Aedes aegypti and Aedes albopictus? Journal of Vector Ecology. 27: 86-95

Lyimo, E.O., Takken, W., Koella, J.C., 1992. Effect of rearing temperature and larval density on larval survival, age at pupation and adult size of Anopheles gambiae. Entomologia Experimentalis et Applicata. 63, 265–271. doi:10.1111/j.1570-7458.1992.tb01583.x

Lyons, C.L., Coetzee, M., Chown, S.L., 2013. Stable and fluctuating temperature effects on the development rate and survival of two malaria vectors, Anopheles arabiensis and Anopheles funestus. Parasites & Vectors. 6, 104. doi:10.1186/1756-3305-6-104

M

Machin, J., Kestler, P., Lampert, G.J., 1991. Simultaneous Measurements of Spiracular and Cuticular Water Losses in Periplaneta Americana: Implications for Whole-Animal Mass Loss Studies. J. Exp. Biol. 161, 439–453.

Machin, J., Lampert, G.J., 1987. An improved water content model for Periplaneta cuticle: Effects of epidermis removal and cuticle damage. J. Insect Physiol. 33, 647–655. doi:10.1016/0022-1910(87)90134-X

Madder, D.J., Surgeoner, G.A., Helson, B.V., 1983. Number of generations, egg production, and developmental time of Culex pipiens and Culex restuans (diptera: culicidae) in southern ontario. Journal of Medical Entomology. 20, 275–287.

Mamai, W., Mouline, K., Blais, C., Larvor, V., Dabiré, K.R., Ouedraogo, G.A., Simard, F., Renault, D., 2014. Metabolomic and ecdysteroid variations in Anopheles gambiae s.l. mosquitoes exposed to the stressful conditions of the dry season in Burkina Faso, West Africa. Physiol. Biochem. Zool. 87, 486–497. doi:10.1086/675697

Mamai, W., Mouline, K., Dabiré, K.R., Ouédraogo, G.A., Renault, D., Simard, F. Mesothoracic spiracles morphology in Anopheles gambiae s.l (Diptera) with particular reference to responses to dry season conditions in Burkina Faso (West Africa). In revision for Parasites and vectors

Marron, M.T., Markow, T.A., Kain, K.J., Gibbs, A.G., 2003. Effects of starvation and desiccation on energy metabolism in desert and mesic Drosophila. J Insect Physiol, 49, 261–270.

McBride, A., Hardie, D.G., 2009. AMP-activated protein kinase--a sensor of glycogen as well as AMP and ATP? Acta Physiol. Oxf. Engl. 196, 99–113. doi:10.1111/j.1748-1716.2009.01975.x

Michaud, M.R., Benoit, J.B., Lopez-Martinez, G., Elnitsky, M.A., Lee Jr., R.E., Denlinger, D.L., 2008. Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, Belgica antarctica. J. Insect Physiol. 54, 645–655. doi:10.1016/j.jinsphys.2008.01.003

Michaud, M.R., Denlinger, D.L., 2004. Molecular modalities of insect cold survival: current understanding and future trends. Int. Congr. Ser., Animals and Environments. Proceedings of the Third International Conference of Comparative Physiology and Biochemistry 1275, 32–46. doi:10.1016/j.ics.2004.08.059

Michaud, M.R., Denlinger, D.L., 2007. Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (Sarcophaga crassipalpis): a metabolomic comparison. J. Comp. Physiol. [B] 177, 753–763.

Minakawa, N., Githure, J.I., Beier, J.C., Yan, G., 2001. Anopheline Mosquito Survival Strategies During the Dry Period in Western Kenya. J. Med. Entomol. 38, 388–392. doi:10.1603/0022-2585-38.3.388

Miner, B.G., Sultan, S.E., Morgan, S.G., Padilla, D.K., Relyea, R.A., 2005. Ecological consequences of phenotypic plasticity. Trends Ecol. Evol. 20, 685–692.

Mitsumasu, K., Kanamori, Y., Fujita, M., Iwata, K., Tanaka, D., Kikuta, S., Watanabe, M., Cornette, R., Okuda, T., Kikawada, T., 2010. Enzymatic control of anhydrobiosis-related accumulation of trehalose in the sleeping chironomid, Polypedilum vanderplanki. FEBS J. 277, 4215–4228. doi:10.1111/j.1742-4658.2010.07811.x

Morales Vargas, R.E., Phumala-Morales, N., Tsunoda, T., Apiwathnasorn, C., Dujardin, J.-P., 2013. The phenetic structure of Aedes albopictus. Infection, Genetics and Evolution. 13, 242–251. doi:10.1016/j.meegid.2012.08.008

Mouline, K., Mamai, W., Agnew, P., Tchonfienet, M., Brengues, C., Dabire, R., Robert, V., Simard, F., 2012. Physiology and development of the M and S molecular forms of Anopheles gambiae in Burkina Faso (West Africa). Medical and Veterinary Entomology. 26, 447–454. doi:10.1111/j.1365-2915.2012.01018.x

Muir, L., Lee, Y.C., 1969. Structures of the D-galactose oligosaccharides from earthworm cuticle collagen. J Biol Chem, 244, 2343–2349.

N

N'Guessan, R., Corbel, V., Akogbeto, M., Rowland, M., 2007. Reduced efficacy of insecticide-treated nets and indoor residual spraying for Malaria control in pyrethroid resistance area, Benin. Emerg. Infect. Dis. 13, 199–206. doi:10.3201/eid1302.060631

Namountougou, M., Simard, F., Baldet, T., Diabaté, A., Ouédraogo, J.B., Martin, T., Dabiré, R.K., 2012. Multiple insecticide resistance in Anopheles gambiae s.l. populations from Burkina Faso, West Africa. PLoS ONE 7, e48412. doi:10.1371/journal.pone.0048412

Navas, C.A., Carvalho, J.E., 2009. Aestivation: Molecular and physiological aspects, 2010 edition. ed. Springer, Berlin ; New York.

Nelson, D.R., Lee Jr., R.E., 2004. Cuticular lipids and desiccation resistance in overwintering larvae of the goldenrod gall fly, Eurosta solidaginis (Diptera: Tephritidae). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 138, 313–320. doi:10.1016/j.cbpc.2004.04.013

Nespolo, R.F., Lardies, M.A., Bozinovic, F., 2003. Intrapopulational variation in the standard metabolic rate of insects: repeatability, thermal dependence and sensitivity (Q10) of oxygen consumption in a cricket. J. Exp. Biol. 206, 4309–4315. doi:10.1242/jeb.00687

Neven, L.G., 2000. Physiological responses of insects to heat. Postharvest Biol. Technol. 21, 103–111. doi:10.1016/S0925-5214(00)00169-1

Neville, A.C., 1975. Biology of the arthropod cuticle. Springer-Verlag, Berlin & New York.

Nielsen, M.G., Elmes, G.W., Kipyatkov, V.E., 1999. Respiratory Q10 varies between populations of two species of Myrmica ants according to the latitude of their sites. J. Insect Physiol. 45, 559–564. doi:10.1016/S0022-1910(98)00162-0

O

Olsen, S.S., Cazzamali, G., Williamson, M., Grimmelikhuijzen, C.J.P., Hauser, F., 2007. Identification of one capa and two pyrokinin receptors from the malaria mosquito Anopheles gambiae. Biochem. Biophys. Res. Commun. 362, 245–251. doi:10.1016/j.bbrc.2007.06.190

Omer, S.M., Cloudsley-Thompson, J.L., 1970. Survival of female Anopheles gambiae Giles through a 9-month dry season in Sudan. Bull. World Health Organ. 42, 319–330.

P

Paaijmans, K.P., Blanford, S., Bell, A.S., Blanford, J.I., Read, A.F., Thomas, M.B., 2010. Influence of climate on malaria transmission depends on daily temperature variation. PNAS. 107, 15135–15139. doi:10.1073/pnas.1006422107

Paluzzi, J.-P., Park, Y., Nachman, R.J., Orchard, I., 2010. Isolation, expression analysis, and functional characterization of the first antidiuretic hormone receptor in insects. Proc. Natl. Acad. Sci. 107, 10290–10295. doi:10.1073/pnas.1003666107

Park, Y., Kim, Y.-J., Adams, M.E., 2002. Identification of G protein-coupled receptors for Drosophila PRXamide peptides, CCAP, corazonin, and AKH supports a theory of ligand-receptor coevolution. Proc. Natl. Acad. Sci. U. S. A. 99, 11423–11428. doi:10.1073/pnas.162276199

Parsell, D.A., Lindquist, S., 1993. The Function of Heat-Shock Proteins in Stress Tolerance: Degradation and Reactivation of Damaged Proteins. Annu. Rev. Genet. 27, 437–496. doi:10.1146/annurev.ge.27.120193.002253

Parsons, P.A., 1991. Evolutionary Rates: Stress and Species Boundaries. Annu. Rev. Ecol. Syst. 22, 1–18. doi:10.1146/annurev.es.22.110191.000245

Parsons, P.A., 2005. Environments and evolution: interactions between stress, resource inadequacy and energetic efficiency. Biol. Rev. Camb. Philos. Soc. 80, 589–610. doi:10.1017/S1464793105006822

Patrick, M.L., Bradley, T.J., 2000. The physiology of salinity tolerance in larvae of two species of Culex mosquitoes: the role of compatible solutes. J. Exp. Biol. 203, 821–830.

Patz, J.A., Martens, W.J., Focks, D.A., Jetten, T.H., 1998. Dengue fever epidemic potential as projected by general circulation models of global climate change. Environ. Health Perspect. 106, 147.

Pétavy, G., David, J.R., Debat, V., Gibert, P., Moreteau, B., 2004. Specific effects of cycling stressful temperatures upon phenotypic and genetic variability of size traits in Drosophila melanogaster. Evolutionary Ecology Research. 6, 873-90.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515. doi:10.1023/B:BILE.0000019559.84305.47

Philip, B.N., Yi, S.-X., Elnitsky, M.A., Lee, R.E., 2008. Aquaporins play a role in desiccation and freeze tolerance in larvae of the goldenrod gall fly, Eurosta solidaginis. J. Exp. Biol. 211, 1114–1119. doi:10.1242/jeb.016758

Pickett, S.T.A., White, P.S., 1985. The Ecology of Natural Disturbance and Patch Dynamics. Academic Press.

Pigliucci, M., 1996. How Organisms Respond to Environmental Changes: From Phenotypes to Molecules (and Vice Versa). Trends Ecol. Evol. 11, 168–173.

Pigliucci, M., 2001. Phenotypic Plasticity: Beyond Nature and Nurture. JHU Press.

Pollock, V.P., McGettigan, J., Cabrero, P., Maudlin, I.M., Dow, J.A.T., Davies, S.-A., 2004. Conservation of capa peptide-induced nitric oxide signalling in Diptera. J. Exp. Biol. 207, 4135–4145. doi:10.1242/jeb.01255

Prince, G., Parsons, P., 1977. Adaptive behaviour of Drosophila adults in relation to temperature and humidity. Aust. J. Zool. 25, 285–290.

Q

Quinlan, M., Hadley, N., 1993. Gas-exchange, ventilatory patterns, and water-loss in 2 lubber grasshoppers - quantifying cuticular and respiratory transpiration. Physiol. Zool. 66, 628–642.

Quinlan, M.C., Gibbs, A.G., 2006. Discontinuous gas exchange in insects. Respir. Physiol. Neurobiol. 154, 18–29. doi:10.1016/j.resp.2006.04.004

Quinlan, M.C., Lighton, J.R.B., 1999. Respiratory physiology and water relations of three species of Pogonomyrmex harvester ants (Hymenoptera: Formicidae). Physiol. Entomol. 24, 293–302. doi:10.1046/j.1365-3032.1999.00140.x

R

R Development Core Team, 2008. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, ISBN 3-900051-07-0, <http://www.R-project.org>.

Ramsay, J.A., 1964. The Rectal complex of the mealworm Tenebrio molitor, L. (Coleoptera, Tenebrionidae). Philos. Trans. R. Soc. Lond. B. Biol. Sci. 248, 279–314. doi:10.1098/rstb.1964.0013

Rathinasabapathi, B., 2000. Metabolic engineering for stress tolerance: installing osmoprotectant synthesis pathways. Ann. Bot. 86, 709–716. doi:10.1006/anbo.2000.1254

Reisen, W.K., Thiemann, T., Barker, C.M., Lu, H., Carroll, B., Fang, Y., Lothrop, H.D., 2010. Effects of warm winter temperature on the abundance and gonotrophic activity of Culex (Diptera: Culicidae) in California. Journal of Medical Entomology. 47, 230–237.

Reiskind, M.H., Zarrabi, A.A., 2012. Is bigger really bigger? Differential responses to temperature in measures of body size of the mosquito, Aedes albopictus. Journal of Insect Physiology. 58, 911–917. doi:10.1016/j.jinsphys.2012.04.006

Renault, H., Roussel, V., Amrani, A.E., Arzel, M., Renault, D., Bouchereau, A., Deleu, C., 2010. The Arabidopsis pop2-1 mutant reveals the involvement of GABA transaminase in salt stress tolerance. BMC Plant Biology, 10, 20.

Richards, C.L., Bossdorf, O., Muth, N.Z., Gurevitch, J., Pigliucci, M., 2006. Jack of all trades, master of some? On the role of phenotypic plasticity in plant invasions. Ecol. Lett. 9, 981–993. doi:10.1111/j.1461-0248.2006.00950.x

Riehle, M.A., Garczynski, S.F., Crim, J.W., Hill, C.A., Brown, M.R., 2002. Neuropeptides and Peptide Hormones in Anopheles gambiae. Science 298, 172–175. doi:10.1126/science.1076827

Rinehart, J.P., Hayward, S.A.L., Elnitsky, M.A., Sandro, L.H., Lee, R.E., Denlinger, D.L., 2006. Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. Proc. Natl. Acad. Sci. 103, 14223–14227. doi:10.1073/pnas.0606840103

Rinehart, J.P., Li, A., Yocum, G.D., Robich, R.M., Hayward, S.A.L., Denlinger, D.L., 2007. Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proc. Natl. Acad. Sci. 104, 11130–11137. doi:10.1073/pnas.0703538104

Rocca, K.A.C., Gray, E.M., Costantini, C., Besansky, N.J., 2009. 2La chromosomal inversion enhances thermal tolerance of Anopheles gambiae larvae. Malaria J, 8, 147. doi: 10.1186/1475-2875-8-147.

Roggero,A., d'Entrèves, P.P., 2005 Geometric morphometric analysis of wing variation between two populations of the Scythris obscurella species-group: geographic or interspecific differences? (Lepidoptera: Scythrididae). SHILAP Revista de Lepidopterología. 33: 101-112.

Rogowitz, G.L., Chappell, M.A., 2000. Energy metabolism of eucalyptus-boring beetles at rest and during locomotion: gender makes a difference. J. Exp. Biol. 203, 1131–1139.

Rohlf, F.J., Slice, D., 1990. Extensions of the procrustes method for the optimal superimposition of landmarks. Systematic Biology. 39, 40–59. doi:10.2307/2992207

Rosenfeld, J., Capdevielle, J., Guillemot, J.C., Ferrara, P., 1992. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Anal Biochem, 203, 173–179.

Rourke, B.C., 2000. Geographic and altitudinal variation in water balance and metabolic rate in a California grasshopper, Melanoplus sanguinipes. J. Exp. Biol. 203, 2699–2712.

Rowley, M., Hanson, F., 2007. Humidity detection and hygropreference behavior in larvae of the tobacco hornworm, Manduca sexta. J. Insect Sci. 7, 1–10. doi:10.1673/031.007.3901

Rudolph, A.S., Crowe, J.H., 1985. Membrane stabilization during freezing: The role of two natural cryoprotectants, trehalose and proline. Cryobiology 22, 367–377. doi:10.1016/0011-2240(85)90184-1

Rueda, L.M., Patel, K.J., Axtell, R.C., Stinner, R.E., 1990. Temperature-dependent development and survival rates of Culex quinquefasciatus and Aedes aegypti (Diptera: Culicidae) Journal of Medical Entomology. 27:892–898.

S

Sadeghi, S., Adriaens, D., Dumont, H.J., 2009. Geometric morphometric analysis of wing shape variation in ten European populations of Calopteryx splendens (Harris, 1782)(Zygoptera: Odonata). Odonatologica. 38, 343–360.

Saini, R.S., 1964. Histology and physiology of the cryptonephridial system of insects. Trans. R. Entomol. Soc. Lond. 116, 347–392. doi:10.1111/j.1365-2311.1964.tb02302.x

Santolamazza, F., Mancini, E., Simard, F., Qi, Y., Tu, Z., della Torre, A., 2008. Insertion polymorphisms of SINE200 retrotransposons within speciation islands of Anopheles gambiae molecular forms. Malaria J, 7, 163. doi:10.1186/1475-2875-7-163

Savriama, Y., Gómez, J.M., Perfectti, F., Klingenberg, C.P., 2012. Geometric morphometrics of corolla shape: dissecting components of symmetric and asymmetric variation in Erysimum mediohispanicum (Brassicaceae). New Phytologist. 196,945–954.

Scaraffia, P.Y., Wells, M.A., 2003. Proline can be utilized as an energy substrate during flight of Aedes aegypti females. J. Insect Physiol. 49, 591–601. doi:10.1016/S0022-1910(03)00031-3

Schilman, P.E., Waters, J.S., Harrison, J.F., Lighton, J.R.B., 2011. Effects of temperature on responses to anoxia and oxygen reperfusion in Drosophila melanogaster. J. Exp. Biol. 214, 1271–1275. doi:10.1242/jeb.052357

Schlichting, C.D., Pigliucci, M., 1998. Phenotypic evolution: a reaction norm perspective. xii + 387 pp.

Schmidt-Nielsen, K., 1990. Animal Physiology Adaptation and Environment. Zoology, Cambridge University Press 4th Ed.

Schmidt-Nielsen, K., 1997. Animal physiology: Adaptation and environment. Cambridge University Press.

Siaussat, D., Laparie, M., Maria, A., Renault, D., 2013. Heat shock protein responses to salinity, food deprivation, and temperature in the invasive ground beetle Merizodus soledadinus at the Kerguelen Islands. Polar Biol. 36, 201–209. doi:10.1007/s00300-012-1252-x

Simard, F., Lehmann, T., Lemasson, J.J., Diatta, M., Fontenille, D., 2000. Persistence of Anopheles arabiensis during the severe dry season conditions in Senegal: an indirect approach using microsatellite loci. Insect Mol Biol, 9, 467–479. doi: 10.1046/j.1365-2583.2000.00210.x

Simard, F., Lehmann, T., Lemasson, J.J., Diatta, M., Fontenille, D., 2001. Persistence of Anopheles arabiensis during the severe dry season conditions in Senegal: an indirect approach using microsatellite loci. Insect Mol. Biol. 9, 467–479.

Simon, P., 2003. Q-Gene: processing quantitative real-time RT–PCR data. Bioinformatics 19, 1439–1440. doi:10.1093/bioinformatics/btg157

Sinclair, B.J., Chown, S.L., 2003. Rapid responses to high temperature and desiccation but not to low temperature in the freeze tolerant sub-Antarctic caterpillar Pringleophaga marioni (Lepidoptera, Tineidae). J. Insect Physiol. 49, 45–52. doi:10.1016/S00

Sinclair, B.J., Gibbs, A.G., Roberts, S.P., 2007. Gene transcription during exposure to, and recovery from, cold and desiccation stress in Drosophila melanogaster. Insect Molecular Biology 16, 435–443. doi:10.1111/j.1365-2583.2007.00739.x

Smith, K.E., Raymond, S.L., Valenti, M.L., Smith, P.J.S., Linser, P.J., 2010. Physiological and pharmacological characterizations of the larval Anopheles albimanus rectum support a change in protein distribution and/or function in varying salinities. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 157, 55–62. doi:10.1016/j.cbpa.2010.05.002

Smith, K.E., VanEkeris, L.A., Okech, B.A., Harvey, W.R., Linser, P.J., 2008. Larval anopheline mosquito recta exhibit a dramatic change in localization patterns of ion transport proteins in response to shifting salinity: a comparison between anopheline and culicine larvae. J. Exp. Biol. 211, 3067–3076. doi:10.1242/jeb.019299

Socha, R., Šula, J., 2006. Flight muscles polymorphism in a flightless bug, Pyrrhocoris apterus (L.): Developmental pattern, biochemical profile and endocrine control. J. Insect Physiol. 52, 231–239. doi:10.1016/j.jinsphys.2005.10.009

Sørensen, J.G., Loeschcke, V., 2007. Studying stress responses in the post-genomic era: its ecological and evolutionary role. J. Biosci. 32, 447–456. doi:10.1007/s12038-007-0044-x

Soto, I.M., Carreira, V.P., Soto, E.M., Hasson, E., 2007. Wing morphology and fluctuating asymmetry depend on the host plant in cactophilic Drosophila. Journal of Evolutionary Biology. 21, 598–609.

Stankiewicz, B., Van Bergen, P.F., Duncan, I.J., Carter, J.F., Briggs, D.E.G., Evershed, R.P., 1996. Recognition of chitin and proteins in invertebrate cuticles using analytical pyrolysis/gas

chromatography and pyrolysis/gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom, 10, 1747–1757.

Steele, J.E., Paul, T., 1985. Corpus cardiacum stimulated trehalose efflux from cockroach (Periplaneta americana) fat body: control by calcium. Can. J. Zool. 63, 63–66. doi:10.1139/z85-012

Storey, J.M., Storey, K.B., 1985. Triggering of cryoprotectant synthesis by the initiation of ice nucleation in the freeze tolerant frog, Rana sylvatica. J. Comp. Physiol. B 156, 191–195. doi:10.1007/BF00695773

Storey, K.B., Baust, J.G., Storey, J.M., 1981. Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, Eurosta solidaginis. J. Comp. Physiol. 144, 183–190. doi:10.1007/BF00802756

Storey, K.B., Storey, J.M., 1990. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. Q. Rev. Biol. 65, 145–174.

Storey, K.B., Storey, J.M., 1991. Biochemistry of cryoprotectants, in: Jr, R.E.L., Denlinger, D.L. (Eds.), Insects at low temperature. Springer US, pp. 64–93.

Storey, K.B., Storey, J.M., 2007. Tribute to P. L. Lutz: putting life on "pause" – molecular regulation of hypometabolism. J. Exp. Biol. 210, 1700–1714. doi:10.1242/jeb.02716

Storey, K.B., Storey, J.M., 2012. Insect cold hardiness: metabolic, gene, and protein adaptation1This review is part of a virtual symposium on recent advances in understanding a variety of complex regulatory processes in insect physiology and endocrinology, including development, metabolism, cold hardiness, food intake and digestion, and diuresis, through the use of omics technologies in the postgenomic era. Can. J. Zool. 90, 456–475. doi:10.1139/z2012-011

Sutcliffe, D.W., 1962. The Composition of Haemolymth in Aquatic Insects. J. Exp. Biol. 39, 325–344.

T

Tanaka, S., Wolda, H., Denlinger, D.L., 1988. Group size affects the metabolic rate of a tropical beetle. Physiol. Entomol. 13, 239–241. doi:10.1111/j.1365-3032.1988.tb00928.x

Taylor, C.E., Toure, Y.T., Coluzzi, M., Petrarca, V., 1993. Effective population size and persistence of Anopheles arabiensis during the dry season in West Africa. Med Vet Entomol, 7, 351–357. doi: 10.1111/j.1365-2915.1993.tb00704.x

Teets, N.M., Denlinger, D.L., 2013. Physiological mechanisms of seasonal and rapid cold-hardening in insects. Physiol. Entomol. 38, 105–116. doi:10.1111/phen.12019

Teets, N.M., Kawarasaki, Y., Jr, R.E.L., Denlinger, D.L., 2013. Expression of genes involved in energy mobilization and osmoprotectant synthesis during thermal and dehydration stress in the Antarctic midge, Belgica antarctica. J. Comp. Physiol. B 183, 189–201. doi:10.1007/s00360-012-0707-2

Teets, N.M., Peyton, J.T., Ragland, G.J., Colinet, H., Renault, D., Hahn, D.A., Denlinger, D.L., 2012. Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. Physiol. Genomics 44, 764–777. doi:10.1152/physiolgenomics.00042.2012

Telfer, W.H., Kunkel, J.G., 1991. The function and evolution of insect storage hexamers. Annu Rev Entomol, 36, 205–228.

Terblanche, J.S., Chown, S.L., 2006. The relative contributions of developmental plasticity and adult acclimation to physiological variation in the tsetse fly, Glossina pallidipes (Diptera, Glossinidae). J. Exp. Biol. 209, 1064–1073. doi:10.1242/jeb.02129

Terblanche, J.S., Sinclair, B.J., Jaco Klok, C., McFarlane, M.L., Chown, S.L., 2005. The effects of acclimation on thermal tolerance, desiccation resistance and metabolic rate in Chirodica chalcoptera (Coleoptera: Chrysomelidae). J. Insect Physiol. 51, 1013–1023.

Terhzaz, S., Cabrero, P., Robben, J.H., Radford, J.C., Hudson, B.D., Milligan, G., Dow, J.A.T., Davies, S.-A., 2012. Mechanism and function of Drosophila capa GPCR: a desiccation stress-responsive receptor with functional homology to human neuromedin receptor. PLoS ONE 7, e29897. doi:10.1371/journal.pone.0029897

Thorat, L.J., Gaikwad, S.M., Nath, B.B., 2012. Trehalose as an indicator of desiccation stress in Drosophila melanogaster larvae: A potential marker of anhydrobiosis. Biochem. Biophys. Res. Commun. 419, 638–642. doi:10.1016/j.bbrc.2012.02.065

Timmermans, M.J.T.N., Roelofs, D., Nota, B., Ylstra, B., Holmstrup, M., 2009. Sugar sweet springtails: on the transcriptional response of Folsomia candida (Collembola) to desiccation stress. Insect Mol. Biol. 18, 737–746. doi:10.1111/j.1365-2583.2009.00916.x

Tolleter, D., Hincha, D.K., Macherel, D., 2010. A mitochondrial late embryogenesis abundant protein stabilizes model membranes in the dry state. Biochim. Biophys. Acta BBA - Biomembr. 1798, 1926–1933. doi:10.1016/j.bbamem.2010.06.029

Touré, Y.T., Petrarca, V., Traoré, S.F., Coulibaly, A., Maïga, H.M., Sankaré, O., Sow, M., Deco, M.A.D., Coluzzi, M., 1994. Ecological genetic studies in the chromosomal form Mopti of Anopheles gambiae s.str. in Mali, West Africa. Genetica 94, 213–223. doi:10.1007/BF01443435

Touré, Y.T., Petrarca, V., Traoré, S.F., Coulibaly, A., Maiga, H.M., Sankaré, O., Sow, M., Di Deco, M.A., Coluzzi, M., 1998. The distribution and inversion polymorphism of chromosomally recognized taxa of the Anopheles gambiae complex in Mali, West Africa. Parassitologia, 40, 477–511.

True, J.R., 2003. Insect melanism: the molecules matter. Trend Ecol Evol, 18, 640–647. doi:10.1016/j.tree.2003.09.006

Truman, J.W., Riddiford, L.M., 1999. The origins of insect metamorphosis. Nat.-Lond.- 447–447.

Tsuda, Y., Kim, K.S., 2008. Sudden autumnal appearance of adult Culex tritaeniorhynchus (Diptera: Culicidae) at a park in urban Tokyo: first field evidence for prediapause migration. Journal of Medical Entomology. 45, 610–616.

U

V

Van der Horst, D.J., 2003. Insect adipokinetic hormones: release and integration of flight energy metabolism. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 136, 217–226.

Van der Horst, D.J., Rodenburg, K.W., 2012. Adipokinetic hormones and their role in lipid mobilization in insects. In Beenakkers, A.M.T., Horst, D.J.V. der, Marrewijk, W.J.A.V., 1981. Role

of Lipids in Energy Metabolism, in: Downer, R.G.H. (Ed.), Energy Metabolism in Insects. Springer US, pp. 53–100.

Verberk, W.C.E.P., Sommer, U., Davidson, R.L., Viant, M.R., 2013. Anaerobic metabolism at thermal extremes: a metabolomic test of the oxygen limitation hypothesis in an aquatic insect. Integr. Comp. Biol. 53, 609–619. doi:10.1093/icb/ict015

Vesala, L., Salminen, T.S., Kostál, V., Zahradnívcková, H., Hoikkala, A., 2012. Myo-inositol as a main metabolite in overwintering flies: seasonal metabolomic profiles and cold stress tolerance in a northern drosophilid fly. J. Exp. Biol. 215, 2891–2897.

Vogel, S. 1981. Life in moving fluids. Princeton: Princeton Univerity Press

W

Wagoner, K.M., Lehmann, T., Huestis, D.L., Ehrmann, B.M., Cech, N.B., Wasserberg, G., 2014. Identification of morphological and chemical markers of dry-and wet-season conditions in female Anopheles gambiae mosquitoes. Parasit. Vectors 7, 294.

West-Eberhard, M.J., 2003. Developmental Plasticity and Evolution. Oxford University Press.

White, B.J., Kundert, P.N., Turissini, D.A., Ekeris, L.V., Linser, P.J., Besansky, N.J., 2013. Dose and developmental responses of Anopheles merus larvae to salinity. J. Exp. Biol. 216, 3433–3441. doi:10.1242/jeb.087189

White, J.F., Torres, M.S., 2010. Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? Physiol. Plant. 138, 440–446. doi:10.1111/j.1399-3054.2009.01332.x

WHO, 2013. Malaria entomology and vector control. World Health Organization.

Wilhelmus, M.M.M., de Waal, R.M.W., Verbeek, M.M., 2007. Heat shock proteins and amateur chaperones in amyloid-Beta accumulation and clearance in Alzheimer's disease. Mol. Neurobiol. 35, 203–216.

Williams, A.E., Bradley, T.J., 1998. The effect of respiratory pattern on water loss in desiccation-resistant Drosophila melanogaster. J. Exp. Biol. 201, 2953–2959.

Williams, A.E., Rose, M.R., Bradley, T.J., 1998. Using laboratory selection for desiccation resistance to examine the relationship between respiratory pattern and water loss in insects. J. Exp. Biol. 201, 2945–2952.

Williams, D.F., Vander Meer, R.K., Lofgren, C.S., 1987. Diet-induced nonmelanized cuticle in workers of the imported fire ant Solenopsis invicta Buren. Arch Insect Biochem Physiol, 4, 251–259. doi: 10.1002/arch.940040403

Williams, J.B., Jr, R.E.L., 2008. Differences in cold tolerance, desiccation resistance, and cryoprotectant production between three populations of Eurosta solidaginis collected from different latitudes. J. Comp. Physiol. B 178, 365–375. doi:10.1007/s00360-007-0229-5

Willis, J.H., Iconomidou, V.A., Smith, R.F., Hamodrakas, S.J., 2005.) Cuticular proteins. In Comprehensive Molecular Insect Science (Gilbert, L.I., Iatrou, K., Gill, S.S., eds), pp. 79–110. Elsevier, Oxford.

Wilps, H., Gäde, G., 1990. Hormonal regulation of carbohydrate metabolism in the blowfly Phormia terraenovae. J. Insect Physiol. 36, 441–449. doi:10.1016/0022-1910(90)90062-K

Withers, P.C., 1992. Comparative animal physiology. Saunders College, Orlando.

Woodring, J., Lorenz, M.W., Hoffmann, K.H., 2002. Sensitivity of larval and adult crickets (Gryllus bimaculatus) to adipokinetic hormone. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 133, 637–644. doi:10.1016/S1095-6433(02)00134-4

Worland, M.R., Grubor-Lajsic, G., Montiel, P.O., 1998. Partial desiccation induced by sub-zero temperatures as a component of the survival strategy of the Arctic collembolan Onychiurus arcticus (Tullberg). J. Insect Physiol. 44, 211–219. doi:10.1016/S0022-1910(97)00166-2

X

Y

Yancey, P.H., 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J Exp Biol, 208, 2819–2830. doi:10.1242/jeb.01730

Yang, P., Tanaka, H., Kuwano, E., Suzuki, K., 2008. A novel cytochrome P450 gene (CYP4G25) of the silkmoth Antheraea yamamai: Cloning and expression pattern in pharate first instar larvae in relation to diapause. J. Insect Physiol. 54, 636–643. doi:10.1016/j.jinsphys.2008.01.001

Yaro, A.S., Traoré, A.I., Huestis, D.L., Adamou, A., Timbiné, S., Kassogué, Y., Diallo, M., Dao, A., Traoré, S.F., Lehmann, T., 2012. Dry season reproductive depression of Anopheles gambiae in the Sahel. Journal of Insect Physiology. 58, 1050–1059. doi:10.1016/j.jinsphys.2012.04.002

Yoder, J.A., Denlinger, D.L., Wolda, H., 1993. Aggregation promotes water conservation during diapause in the tropical fungus, Beetle, Stenotarsus rotundus. Entomol Exp Appl 63:203–205.

Yoder, J.A., Smith, B.E., 1997. Enhanced water conservation in clusters of convergent lady beetles, Hippodamia convergens. Entomol. Exp. Appl. 85, 87–89. doi:10.1046/j.1570-7458.1997.00237.x

Z

Zachariassen, K.E., 1996. The water conserving physiological compromise of desert insects. Eur J Entomol, 93, 359–367.

Zera, A.J., Denno, R.F., 1997. Physiology and ecology of dispersal polymorphism in insects. Annu. Rev. Entomol. 42, 207–230. doi:10.1146/annurev.ento.42.1.207

Zera, A.J., Sall, J., Grudzinski, K., 1997. Flight-muscle polymorphism in the cricket Gryllus firmus: muscle characteristics and their influence on the evolution of flightlessness. Physiol. Biochem. Zool. 70, 519–529. doi:10.1086/515865

Ziegler, R., Ashida, M., Fallon, A.M., Wimer, L.T., Wyatt, S.S., Wyatt, G.R., 1979. Regulation of glycogen phosphorylase in fat body of Cecropia silkmoth pupae. J. Comp. Physiol. 131, 321–332. doi:10.1007/BF00688807

Ziegler, R., Isoe, J., Moore, W., Riehle, M.A., Wells, M.A., 2011. The putative AKH receptor of the tobacco hornworm, Manduca sexta, and its expression. J. Insect Sci. Online 11, 40. doi:10.1673/031.011.0140

Zimmermann, G., Bosc, P., Valade, P., Cornette, R., Améziane, N., Debat, V., 2012. Geometric morphometrics of carapace of Macrobrachium australe (Crustacea: Palaemonidae) from Reunion Island. Acta Zoologica. 93, 492–500. doi:10.1111/j.1463-6395.2011.00524.x

Liste des publications

Article I

Hidalgo, K., Mouline, K., Mamai, W., Foucreau, N., Dabiré, K.R., Bouchereau, A., Simard, F., Renault, D., 2014. Novel insights into the metabolic and biochemical underpinnings assisting dry-season survival in female malaria mosquitoes of the Anopheles gambiae complex. Journal of Insect Physiology. doi:10.1016/j.jinsphys.2014.07.003

Article II

Hidalgo, K., Montazeau, C., Siaussat, D., Braman, V., Simard, F., Renault, D., Mouline, K., (In prep). Metabolic rate and AKH peptides variations in females Anopheles coluzzii and An. gambiae during the dry season. In perp for Physiological and Biochemical Zoology

Article III

Hidalgo, K., Siaussat, D., Braman, V., Mouline, K., Simard, F., Renault, D., Submit. Physiological plasticity of water stress resistance between geographic populations of the malarial mosquito *An. coluzzii*. In perp for Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology

Article IV

Hidalgo, K., Dujardin, J.-P., Mouline, K., Dabiré, R.K., Renault, D., Simard, F., In revision. Seasonal variation in wing size and shape between geographic populations of the malaria vector, Anopheles coluzzii in Burkina-Faso (West Africa). In revision for Acta Tropica

Article V

Hidalgo, K., Laparie, M., Bical, R., Larvor, V., Bouchereau, A., Siaussat, D., Renault, D., 2013. Metabolic fingerprinting of the responses to salinity in the invasive ground beetle Merizodus soledadinus at the Kerguelen Islands. Journal of Insect Physiology. doi:10.1016/j.jinsphys.2012.10.017

Article VI

Khodayari, S., Moharramipour, S., Larvor, V., Hidalgo, K., Renault, D., 2013. Deciphering the Metabolic Changes Associated with Diapause Syndrome and Cold Acclimation in the Two-Spotted Spider Mite Tetranychus urticae. PLoS ONE 8, e54025. doi:10.1371/journal.pone.0054025

Article VII

Foucreau, N., Renault, D., Hidalgo, K., Lugan, R., Pétillon, J., 2012. Effects of diet and salinity on the survival, egg laying and metabolic fingerprints of the ground-dwelling spider Arctosa fulvolineata (Araneae, Lycosidae). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 163, 388–395. doi:10.1016/j.cbpa.2012.07.001

Annexes

1. **Metabolic fingerprintings of the responses to salinity in the invasive ground beetle *Merizodus soledadinus* at the Kergielen Islands (Article V)**

2. **Deciphering the metabolomic changes associated with diapause syndrome and cold acclimation in the two-spotted spider mite *Tetranychus uticae* (Artcile VI)**

3. **Metabolic response to salt stress and diet in the ground-dwelling spider *Arctosa fulvolineta* (Aranae, Lycosidae) (Article VII)**

Metabolic fingerprinting of the responses to salinity in the invasive ground beetle *Merizodus soledadinus* at the Kerguelen Islands - (Article V)

Hidalgo K, Laparie M, Bical R, Larvor V, Bouchereau A, Siaussat D, Renault D

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Abstract

Salinity is an abiotic factor that may impact survival and fitness of terrestrial insects in coastal environments. Meanwhile, some terrestrial arthropods can survive in hypersaline environments, and counterbalance osmotic stress by intra- and extracellular buildups of organic osmolytes. The ground beetle *Merizodus soledadinus* originates from South America and it is distributed in forests and riparian zones, where salinity levels are considerably low. This species has been introduced at the Kerguelen Islands a century ago, where it colonized coastal areas (tide drift lines), and must thus withstand salinity variations due to tide, spray, and organic matter deposited therein. In the present study, we addressed the physiological plasticity of adult *M. soledadinus* to saline conditions, by monitoring body water content and survival in adult *M. soledadinus* experimentally subjected to different salinities. We also investigated possible metabolic adjustments involved at three contrasted salinity levels (0, 35, 70‰) at 4 and 8 °C. We hypothesized that this invasive ground beetle can withstand a broad range of salinity conditions thanks to the plastic accumulation of compatible solutes. The study revealed a progressive drop in body water content in individuals exposed to 35 and 70‰, as opposed to the controls. Metabolic fingerprints showed compatible solute (erythritol, alanine, glycine and proline) accumulation at medium and high salinity conditions (35 and 70‰). We concluded that the osmo-induced accumulation of amino acids and polyols was likely to modulate the ground beetles' body water balance on medium saline substrates, thus enhancing their survival ability and explaining their successful colonization of the tide drift line at the Kerguelen Islands

Keywords: biological invasions, survival, body water content, osmoregulation, compatible solutes, amino acid, glycine, alanine

1. Introduction

Salinity places physiological challenges on terrestrial insects, including osmotic and ionic stresses that disrupt body water volume, biochemical processes, cellular homeostasis, and ionic balance (Witteveen *et al.*, 1987; Sowers *et al.*, 2006). Meanwhile, some terrestrial arthropods can endure and survive saline levels up to 70-80 parts per thousand (Pétillon *et al.*, 2008, 2011) by accumulating several osmolyte classes, including monosaccharides, disaccharides, sugar alcohols, quaternary amino acids (Misra & Gupta, 2005; Yancey, 2005; Wang *et al.*, 2008). Altogether, these compounds contribute to maintain the osmotic balance among body compartments (Benoit, 2010). Due to their physico-chemical properties, osmolytes do not alter intracellular biochemistry and physiology. Indeed, osmolytes are not detrimental to macromolecules even at high concentrations, and have limited effects on pH, charge balance, and enzyme activity (Yancey, 2005).

Inorganic and organic osmolytes have been demonstrated as important contributors for the regulation of hemolymph osmolality during dehydration (Nicolson, 1980). The intra- and extracellular buildup of organic osmolytes is well known to attenuate acute hyperosmotic stress. Hemolymph amino acids have been found to contribute to osmoregulation during dehydration in the tenebrionid beetles, *Onymacris rugatipennis* (Coleoptera= Tenebrionidae) (Naidu, 2001), and *Physadesmia globosa* (Coleoptera= Tenebrionidae) (Naidu, 2006). Patrick & Bradley (2000) demonstrated that hypersalinity stress in the mosquito *Culex tarsalis* (Diptera= Culicidae) larvae was counterbalanced by high proline accumulation in intra- and extracellular compartments, and increased hemolymph trehalose levels. Organic osmolytes, that can act as osmoprotectants, have also been shown to scavenge reactive oxygen species (ROS), which increase under hypersaline conditions as a result of salt-altered membrane potential (Vaidyanathan *et al.*, 2003). Lastly, osmoprotectants were found to stabilize proteins in their natural conformation and were thus termed 'chemical chaperones' in reference to the well-described mode of action of other members of molecular chaperone family (Welch & Brown, 1996).

Merizodus soledadinus Guérin-Méneville (Coleoptera= Carabidae) was first described from the Falkland Islands, but its native range spans from the Falkland

Islands to southern South America (Patagonia) (Jeannel, 1940). A 2009 field survey conducted in Patagonia, together with the scarce literature available on the biology and ecology of the species, indicated that *M. soledadinus* primarily occurs in *Notophagous* sp. forest litter and along rivers in valleys (Roig-Junent & Dominguez, 2001; Siaussat *et al.* in press). By contrast, within the Kerguelen Islands, where it was introduced in 1913 and became invasive (Jeannel, 1940), *M. soledadinus* most often established permanent populations in terrestrial coastal habitats (Ernsting, 1993; Laparie *et al.*, 2010; Lebouvier *et al.*, 2011), and more particularly in intertidal zones. Prey community structure, characterized by the dominance of invertebrate decomposers (Vernon *et al.*, 1998) which specimens of *M. soledadinus* feed on, likely governs this intertidal distribution, as well as microhabitat moisture levels (Lalouette *et al.*, 2012). In these habitats, water remains available permanently, but varies from fresh (rainwat) to hyper saline because of tides, sea-spray and evaporation. Salinity can thus greatly vary, from *ca.* 30 to *ca.* 75 parts per thousand, in intertidal zones (Rudloe, 1979). As a result, a consideration of the native ecological range of *M. soledadinus*, and its invasive success in hyper saline strandlines of the Kerguelen Islands is intriguing. Indeed, most terrestrial species from saline environments can survive in non-saline environments; however examples of species from non-saline habitats colonizing intertidal zones are more rare (Pétillon *et al.*, 2011).

Saline stress effects are well documented in plants (Misra & Gupta, 2005), in fishes (Kefford *et al.*, 2004), in algae (Kirst, 1990) and in marine arthropods (Rhodes-Ondi & Turner, 2010), but few studies have been conducted on terrestrial arthropods (Rhodes-Ondi & Turner, 2010; Pétillon *et al.*, 2008). To gain further insights of the mechanisms that enhanced the colonization success of this alien predator on Kerguelen Islands, we examined the physiological plasticity of adult *M. soledadinus* subjected to a range of experimental saline conditions (0, 35, and 70‰). We hypothesized that the ground beetles exposed to increasing salt concentrations would survive and maintain their body water content constant thanks to an accumulation of compatible solutes. As acclimation to the experimental conditions may occur during the experiments, we also investigated to what extent the kinetics of these adjustments could be time and temperature-dependent.

2. Materials and methods

2.1. Study site and sampling method

Merizodus soledadinus adults were hand-collected from the Kerguelen Islands in the vicinity of Port-aux-Français research station (49°21'S - 70°13'E) in February 2010 (survival experiments and metabolic fingerprinting of the responses to salinity), and in February 2012 (body water content).

These nocturnal insects (Ottesen, 1990; Siaussat *et al.*, in press) were subsequently maintained under controlled conditions at 8 ± 1 °C (R.H. of 70 ± 5%) in the dark for two days to ensure similar thermal regimes prior to the experiments. During this 48-hour period, the ground beetles were also supplied *ad libitum* with water and food. Food consisted of *Fucellia maritima* larvae (Diptera= Anthomyiidae). *Merizodus soledadinus* imagoes were then directly used to assess salinity tolerance.

2.2. Salinity tolerance

Merizodus soledadinus salinity tolerance was determined by placing batches of ten adults in airtight plastic boxes (diameter 8 cm, depth 6 cm), which were half-filled with local sand saturated with water. Three soil salinity level treatments were used as follows= null salinity (sand and mineral water considered as being non-salted, 0‰ salt), medium salinity (sand and mineral water-35‰ salt), and hypersalinity (sand and mineral water-70‰ salt). Two temperature regimes were assessed for each of the salinities (4.0 ± 0.5 °C and 8.0 ± 0.5 °C, dark conditions). Local sand was washed several times with fresh non-saline water until the salinity reached a nearly null (0‰) concentration. The sand was sterilized with an autoclave, subsequently stored at -80 °C for one day before being dried at 60 °C for one week. Soil salinity was monitored with a WET sensor (2 cm deep; specific sand soil calibration) connected to a HH2 moisture meter (both instruments from Delta-T Devices Ltd, U.K.); it was 2.7 ± 0.3 $mS.m^{-1}$ at 0‰ and 114.5 ± 9.5 $mS.m^{-1}$ at 35‰. It was not possible to get the values at 70‰, as they were over the detection limit of our equipment. In all experimental conditions, individuals were starved to avoid biasing measurements due to the presence of food in the digestive tracts. Previous studies showed that negligible adult mortality was observed in *M. soledadinus* following four weeks of starvation (2 deaths

of 28 individuals, Laparie *et al.*, 2012), and that no cannibalistic behavior occurred (Siaussat *et al.*, in press).

2.2.1. Survivorship experiments

For each experimental condition (three saline conditions, two temperatures), five plastic boxes, each containing ten beetles, were used. A total of 300 ground beetles was used to conduct this experiment, and the survival duration of adult *M. soledadinus* was monitored every three days. An additional survival experiment was conducted to examine the level of desiccation tolerance of the ground beetles. Tolerance to desiccation was determined by placing groups of 10 adult *M. soledadinus* in Petri dishes. Two batches of four Petri dishes were placed under controlled conditions in a walking chamber (temperature= 4.0 ± 0.5 °C, 50% R.H., dark conditions). In the first batch, the ground beetles were food- and water-deprived, and in the second batch, the ground beetles were food-deprived but were supplied with water *ad libitum*. Survival was monitored every day, and dead ground beetles were removed. For all survival experiments, lethal time for 50% of the population (Lt_{50}) was determined.

2.2.2. Body water content

Changes in the amount of body water have been monitored over the course of the experiment at the three saline conditions (0, 35 and 70‰). We focused on the starved ground beetles exposed at 4 °C, a temperature that best represent the thermal conditions of the Kerguelen Islands (Lebouvier *et al.*, 2011). Body water amount was determined at T0, after one (T1), two (T2) and four (T4) weeks of exposure to the experimental conditions. For each saline condition, ten plastic boxes were used, each containing ten ground beetles. Fresh mass was measured immediately after collection on 15 non-sexed individuals at T0, T1, T2 and T4 (Sartorius® microbalance, 0.1 mg accuracy) for saline condition (a total of 180 ground beetles were weighed). Then, the specimens were dried for six days at 60 °C to get their dry mass. Water content was calculated from these data and expressed as a percentage of the fresh mass.

2.2.3. Metabolic fingerprinting

Metabolic assays were performed on adult *M. soledadinus* exposed to three salinity conditions (0, 35 and 70‰) and two temperatures (4 and 8 °C). Metabolites were measured in whole body extracts= *(i)* in 'wild' insects (Ground beetles sampled just after they have been maintained for 48 hours under controlled conditions, T0); (*ii*) after two weeks of exposure to experimental conditions (T2); and (*iii*) after four weeks of exposure to experimental conditions (T4). For each experimental condition, ten samples were prepared, each sample comprised of a pool of three randomly collected beetles (*e.g.* sampled in three plastic boxes from each experimental condition) directly plunged in 800 µL of 70° ethanol and stored at -20 °C until analysis. All the sampled beetles were alive at the time of their collection (limb movements were observable). A total of 130 samples were prepared.

2.2.3.1 Sample preparation and derivatization

The samples were vacuum-dried (Speed Vac Concentrator, MiVac, Genevac Ltd., Ipswich, England), and re-disolved in 600 µL of methanol-chloroform (2=1). Samples were homogenized using a bead-beating device (Retsch^{TM} MM301, Retsch GbmH, Haan, Germany) at 25 Hz for 1.5 min. Then, 400 µL of ice-cold ultrapure water was added, and each sample was vortexed. Following centrifugation at 8,000 *g* for 10 min at 4 °C, 210 µL aliquots of the upper aqueous phase, which contained polar metabolites, were transferred to microtubes and vacuum-dried (Speed Vac Concentrator, MiVac, Genevac Ltd., Ipswich, England). The polar phase aliquots were resuspended in 15 µL of 20 mg.mL^{-1} methoxyaminehydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine prior to incubation under orbital shaking at 30 °C for 60 min. Following incubation, 15 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma, #394866) was added, and derivatization was conducted at 37 °C for 30 min.

2.2.3.2 Metabolite analysis

We measured metabolites using a Trace GC Ultra chromatograph with flame ionization as the detector (GC-FID, Thermo Fischer Scientific Inc, Waltham, MA, USA). The injector temperature was held at 260 °C. The oven temperature ranged

from 70 to 147 °C at 9 °C.min^{-1}, from 147 to 158 °C at 0.5 °C.min^{-1}, from 158 to 300 °C at 5.0 °C.min^{-1}, and remained for 3 min at 300 °C. A 30m fused silica column (DB-5, 30 m x 0.320 mm x 0.25 µm, 5%-Phenyl-methylpolysiloxane, 95% dimethylpolysiloxane) was used, with helium as the carrier gas at a rate of 1 ml.min^{-1}. One microliter of each sample was injected using the splitless mode (25=1). Randomized sample sequences were established for sample injection, and each sequence was initiated with a quality control. Standard samples consisting of 60 pure reference compounds at 200 and 300 µM concentrations were run. Chromatograms were deconvoluted using ChemStation v2.0.7, and metabolite levels were quantified using arabinose as internal standard and by comparison with individual external standards.

To ensure the reliability of our data and the accuracy of peak annotation, additional runs were conducted using a GC-MS (Dittami *et al.*, 2011). It consisted of a CTC CombiPal autosampler (GERSTEL GmbH & Co.KG, Mülheim an der Ruhr), a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). The injector and oven settings were identical those described above, and the column used was a 30m fused silica column (TR5 MS, I.D. 25 mm, 95% dimethyl siloxane, 5% Phenyl Polysilphenylene-siloxane).

2.3. Statistical analysis

Using *Minitab* 12.2 statistical software, probit regression models and Pearson correlation tests were processed to measure the lethal times for 50% of the population (Lt_{50}) of *M. soledadinus* under each experimental condition. An analysis of covariance (ANCOVA) with *post hoc* comparisons was performed to address the effects of salinity and exposure duration on the body water content of *M. soledadinus*, with body mass as covariable.

Metabolic differences among the 13 following classes of samples were evaluated= wild ground beetles, ground beetles exposed at 4 °C (0, 35 and 70‰ salinities), and ground beetles exposed at 8 °C (0, 35 and 70‰ salinities), either for two or four weeks. Among sugars, polyols, and amino and organic acids, 41 compounds were identified; therefore multivariate methods were employed for data

analysis. Compounds beyond detection limits were eliminated from the analysis. Compounds exceeding a 80% correlation with other compounds were discarded to avoid redundancy and an overestimate in differences among classes in multivariate analyses. Using log-transformed data, we then performed MANOVAs to address physiological differences among the 12 classes of individuals exposed to controlled conditions. Wild individuals were excluded from these MANOVAs to ensure that the differences found did not result from the contrast usually found in the literature between wild individuals and individuals maintained under controlled conditions. As the temperature effect was highly significant (see results section), we decided to analyze individuals maintained at 4 and 8 °C separately. The four-week exposure to 70‰ salinity at 8 °C resulted in a comparatively high mortality, this group was therefore excluded from multivariate analyses to avoid biasing the results with the small sample of extreme individuals that survived. Together with multivariate data, three-way ANOVAs (Salinity, Temperature, and Time) were detailed on individual molecules, and followed when required with Tukey *post-hoc* procedure among levels of significant factors. *P* values were adjusted using the Benjamini and Hochberg algorithm (Benjamini & Hochberg 1995) to control the FDR (α threshold = 0.05).

Class separation was subsequently investigated in Linear Discriminant Analyses (LDA). The wild individuals were included in these analyses so that the physiological dynamics from T0 (natural conditions) to T4 (four weeks of exposure to controlled conditions) has been addressed. Statistical significance of LDAs was checked by using permutation tests (10, 000 permutations). Statistical procedures were conducted with *R* 2.13.1 statistical software (R Development Core Team 2008).

3. Results

3.1. Survivorship experiments

Lethal times for 50% of the population (Lt_{50}) did not differ between the two thermal conditions (4 and 8 °C). Lt_{50} was significantly reduced with increased salinity levels, from 27.0 ± 2.1 days at 35‰ to 19.5 ± 1.4 days at 70‰ at 4 °C; and from 21.4 ± 1.3 days at 35‰ to 15.9 ± 1.7 days at 70‰ at 8 °C ($p < 0.05$). Survival duration (Lt_{50}) of the food- and water-deprived ground beetles was 14.8 ± 0.8 days; it was significantly

lower from the survival durations of the ground beetles from all other experimental conditions ($p < 0.05$), except the ground beetles exposed at 70‰ at 8 °C ($p > 0.05$). Following four weeks of experiment, non-saline conditions did not result in mortality (the ground beetles were food deprived but supplied with water *ad libitum*).

3.2. Body water content and dry mass

Differences in the body water content were detected among groups of individuals exposed to different saline conditions and exposure duration (**Table 1**). Body water content was not different among saline conditions during the first week of the experiment (**Fig. 1A**). However, differences appeared afterwards with water contents being decreased in the ground beetles exposed at 35 and 70‰ whereas it remained constant over the course of the experiment in adult *M. soledadinus* exposed at the non-saline conditions (**Fig. 1A**). Body water content of the individuals exposed at 70‰ was reduced significantly after two weeks of exposure (T2). After 4 weeks of experiment, the body water content of the ground beetles exposed at 35 and 70 ‰ was similar, and the water mass of these beetles was reduced by 28% compared to the control (0‰). Dry masses of adult *M. soledadinus* did not differ significantly among the experimental groups (**Fig. 1B**).

Table 1 Results of the Manova performed in adult *Merizodus soledadinus* for the effects of salinity, duration of exposure, temperature, and their interaction. All numeric data were rounded to two decimal digits. Pillai trace was multiplied by 10. Bold *p* values are significant to the threshold < 0.05.

Data set	Effect	F_{df}	Pillai	*p*
Laboratory individuals	Salinity	2.27_{52}	9.52	$\mathbf{9.37^{10\ -5}}$
(*N* = 100 samples)	Duration	3.66_{26}	6.69	$\mathbf{1.23^{10-5}}$
	Temperature	4.97_{26}	5.98	$\mathbf{8.85^{10-8}}$
	Salinity * Duration	1.68_{52}	8.05	$\mathbf{9.46^{10-3}}$
	Salinity *Temperature	0.64_{52}	6.40	0.23
	Duration *Temperature	1.03_{26}	2.96	0.44
	Salinity * Duration *Temperature	0.53_{26}	1.77	0.96

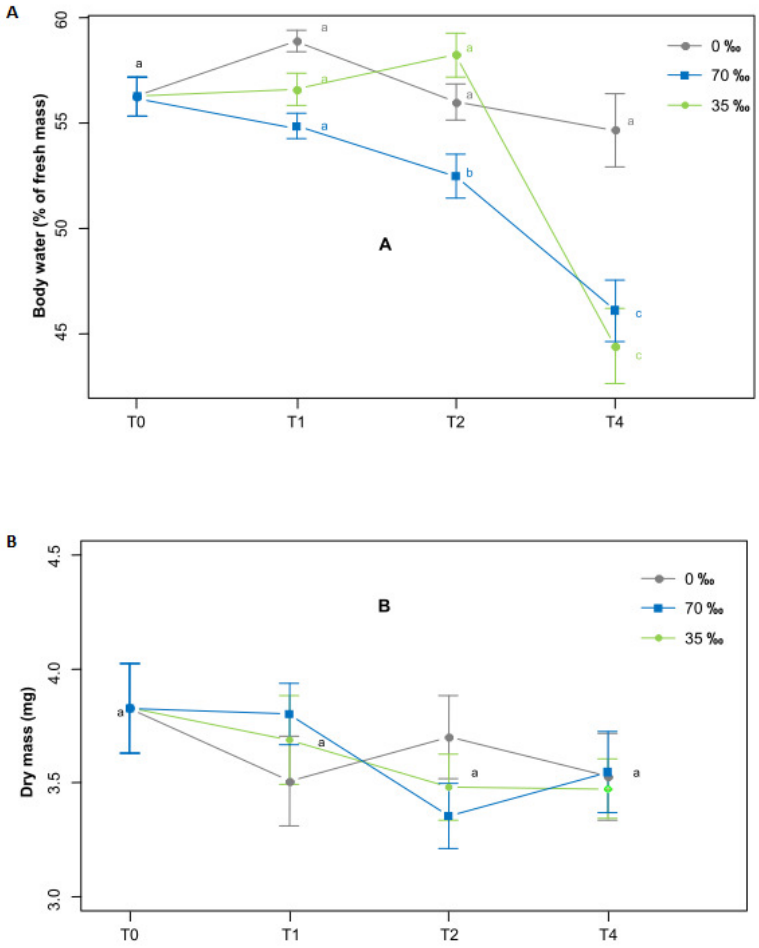

Fig. 1 Comparison of the body water content (A) and dry mass (B) of adult *Merizodus soledadinus* exposed at 0‰, 35‰ and 70‰ salinity for 4 weeks. T0= wild samples, T1= one-week exposed ground beetles, T2= two-week exposed ground beetles, and T4= 4 week exposed ground beetles. Values are means S.E. Distinct letters denote significant differences ($p < 0.05$).

3.3. Metabolic profiles

Physiological differences were detected among groups of individuals exposed to different saline conditions (**Table 2**), temperatures, and exposure duration. The substrate salinity x duration of exposure interaction was also significant.

3.3.1. Responses to saline conditions at 4 °C

The metabolic profiles of individuals maintained at 4° C significantly differed among the three saline conditions (F_{52} = 1.50, p < 0.05), and over the course of the experiment (F_{26} = 2.30, p < 0.05). However, the metabolite level changes over time were not significantly different among the three saline conditions (no interaction, F_{52} = 1.03, p > 0.05). The total compound concentrations (two- and four-week ground beetles pooled) ranged from 111.5 ± 10.6 nmol.mg^{-1} of dry mass (on average ± SE) in

ground beetles exposed at 0‰ to 190.3 ± 24.5 nmol.mg^{-1} of dry mass at 70‰, with ground beetles exposed at 35‰ having an intermediary concentration of 147.6 ± 23.0 nmol.mg^{-1} of dry mass. The total amounts of sugars and free amino acids are presented in **Fig. 2**.

The groups (including wild individuals) showed significant ($p < 0.001$) separation in the Linear Discriminant Analysis (LDA) (**Fig. 3**). The first axis (LD1) accounted for 42.0% of the total inertia, and the between-class inertia was 6 .2 times higher than the within-class inertia. LD1 was primarily characterized by variation in erythritol, alanine, proline, and to a lesser extent, pipecolate, glycine and ethanolamine, sorting salinity levels from 0 to 70‰ by increasing concentrations of the three metabolites (**Fig. 4**). At 70‰, the compound concentrations were higher in four-week relative to two-week exposed individuals.

The second axis (LD2) accounted for 24.7% of the total inertia, and the between-class inertia was 4.1 times higher than the within-class inertia (**Fig. 3**). LD2 was primarily characterized by variation in gluconolactone, mannose, glycerol, glucose, putrescine, and citrate. These metabolites had the highest levels in wild ground beetles, and the lowest levels in four-week exposed ground beetles. Within each saline condition, differences between the two sampling dates increased with increased salinity.

Table 2 Results of the ANCOVA performed in adult *Merizodus soledadinus* for the effects of salinity, exposure duration, dry mass, and their interactions. All numeric data were rounded to two decimal digits. Bold *p* values are significant to the threshold < 0.05.

Dataset	Effect	F value	df	*p value*
Laboratory individuals (N = 150 samples)	Salinity	29.57	1	**1.93^{10-7}**
	Exposure duration	58.07	3	**$< 2.00^{10-16}$**
	Dry mass	90.65	1	**2.00^{10-16}**
	Salinity * Exposure duration	5.06	3	**2.22^{10-3}**
	Exposure duration *Dry mass	5.84	3	**8.12^{10-12}**
	Salinity *Dry mass	0.49	1	0.48
	Salinity * Exposure duration *Dry mass	0.09	3	0.96

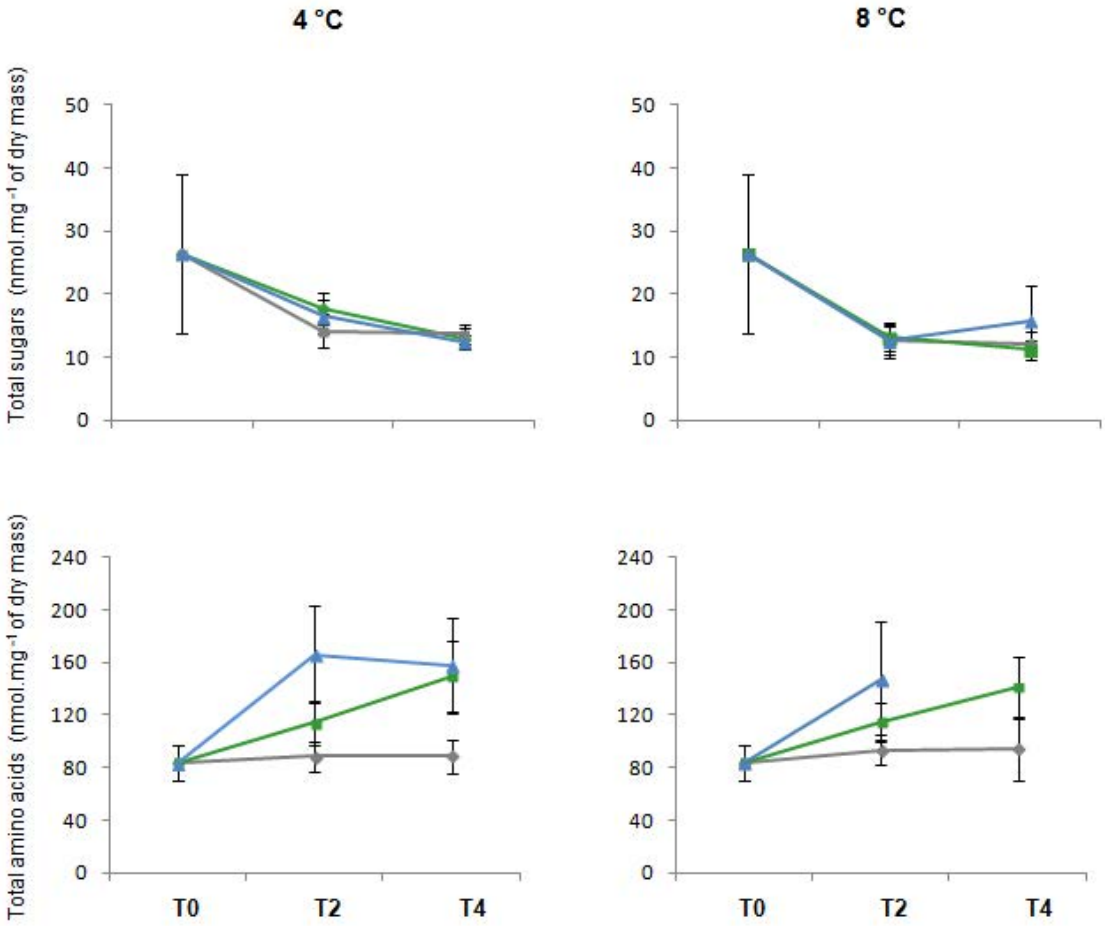

Fig. 2 Temporal variations of total sugar and total amino acid amounts (means ± S.E.) in adult *Merizodus soledadinus* held at 0‰ (grey), 35‰ (green) or 70‰ (blue) salinities and at 4 °C and 8 °C. T0= wild ground beetles, T2= two-week exposed ground beetles, and T4= four-week exposed ground beetles. No significant difference was found ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

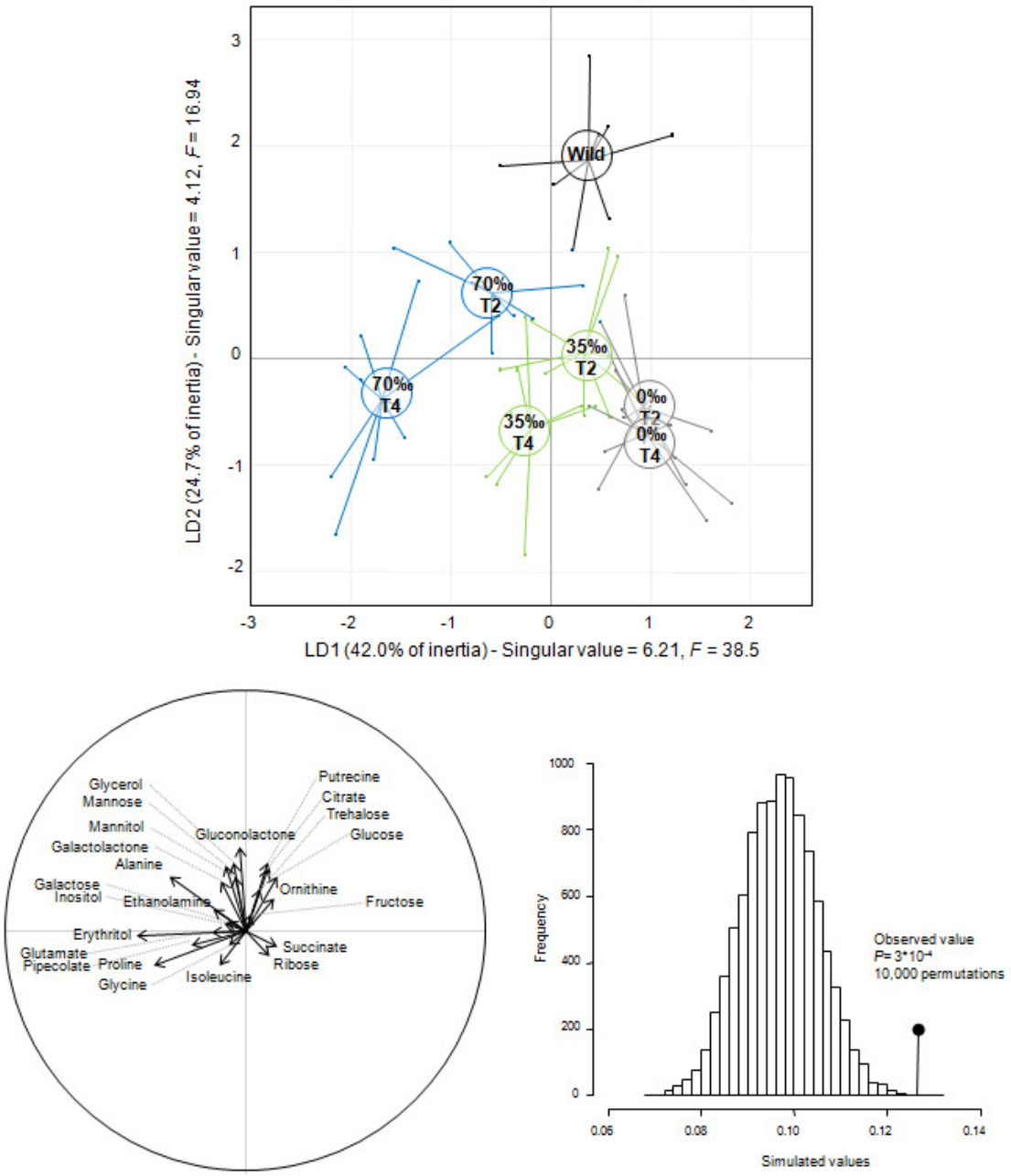

Fig. 3 Sample projection (63 samples) onto the first LDA discriminant plane if the individuals maintained at 4 °C. The singular values are the ratio of between-class and within-class inertias. The lines link the samples (three individuals per sample) to the centroid of their class. Black= wild ground beetles; grey= null salinity (0‰); green= medium salinity (35‰); blue= high salinity (70‰). T0= wild ground beetles, T2= two-week exposed ground beetles, and T4= four-week exposed ground beetles. The correlations circle depicts the normed relation (from −1 to 1) between each compound and linear discriminant axes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

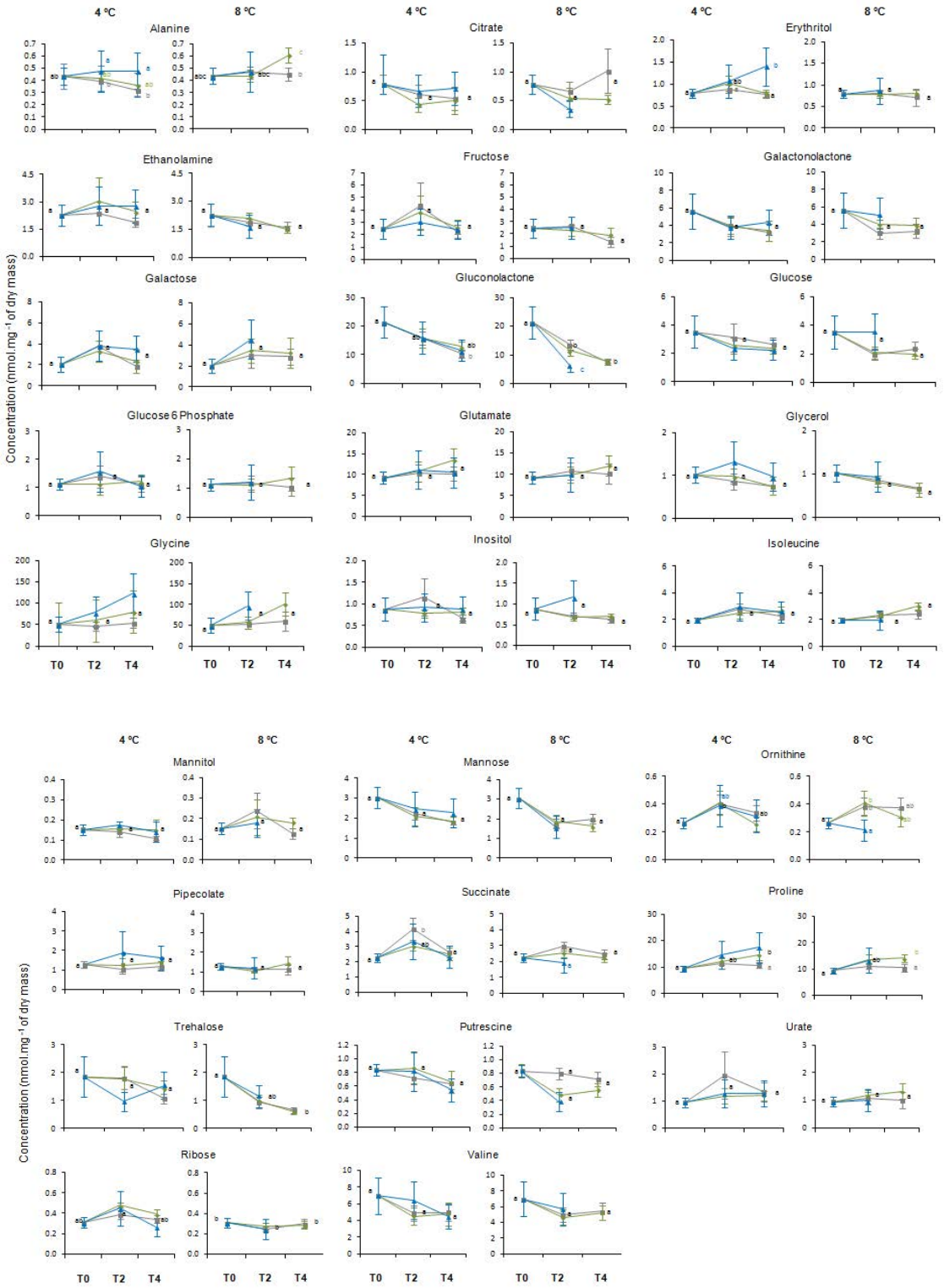

Fig. 4 Temporal variations of metabolite concentrations (means ± S.E.) in adult Merizodus soledadinus held at 0‰ (grey), 35‰ (green) or 70‰ (blue) salinities and at 4 °C and 8 °C. T0= wild ground beetles, T2= two-week exposed ground beetles, and T4= four-week exposed ground beetles. Distinct letters indicate significant differences among the experimental conditions ($p < 0.05$). Black letters mean that the statistical results can be applied for the three saline conditions; no letter means no significant difference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2. Responses to saline conditions at 8 °C

Individuals exposed for four weeks to 70‰ salinity were dead and thus excluded from the analysis. Differences in metabolic profiles among ground beetle groups exposed to the three saline conditions (F_{52} = 2.13, $p < 0.01$) were detected over the course of the experiment (F_{26} = 3.17, $p < 0.01$). The metabolite level also changed over time significantly among the three saline conditions (F_{26} = 2.38, $p < 0.01$). The highest total compound concentrations (two- and four-week ground beetles pooled) were found in ground beetles exposed at 70‰ (177.8 ± 45.8 nmol.mg^{-1} of dry mass on average ± SE), followed by those exposed at 35‰ (164.8 ± 24.5 nmol.mg^{-1} of dry mass) and 0 ‰ (107.5 ± 10.6 nmol.mg^{-1} of dry mass). The total amounts of sugars and free amino acids are presented in **Fig.2**.

The groups, including wild ground beetles, exhibited significant ($p < 0.001$) separation in LDA (**Fig. 5**). The first axis (LD1) accounted for 45.9% of the total inertia, and the between-class inertia was 7.3 times higher than the within-class inertia. LD1 showed separation in the three salinity levels, and the two exposure durations at salinity levels of 35‰. The increased exposure duration at 35‰ induced increased concentrations of alanine, pipecolate, galactolactone, proline, erythritol, glycine and mannitol and decreased levels of ornithine, gluconolactone, citrate, putrescine and succinate (**Fig. 4**). Two-week exposed ground beetles at 35‰ overlapped with 0‰ samples, and two-week exposed ground beetles at 70‰ had similar metabotypes with four-week exposed ones at 35‰. LD2 accounted for 30.6% of the total inertia, and between-class inertia was 5.5 times higher than within-class inertia (**Fig. 5**). LD2 mainly discriminated wild samples for experimental samples. Decreased levels of trehalose, glycerol, fructose, mannose, citrate, gluconolactone, and putrescine characterized starved ground beetles. In a lesser extent, LD2 separated two-week exposed ground beetles at 70‰ and four-week exposed ones at 35‰.

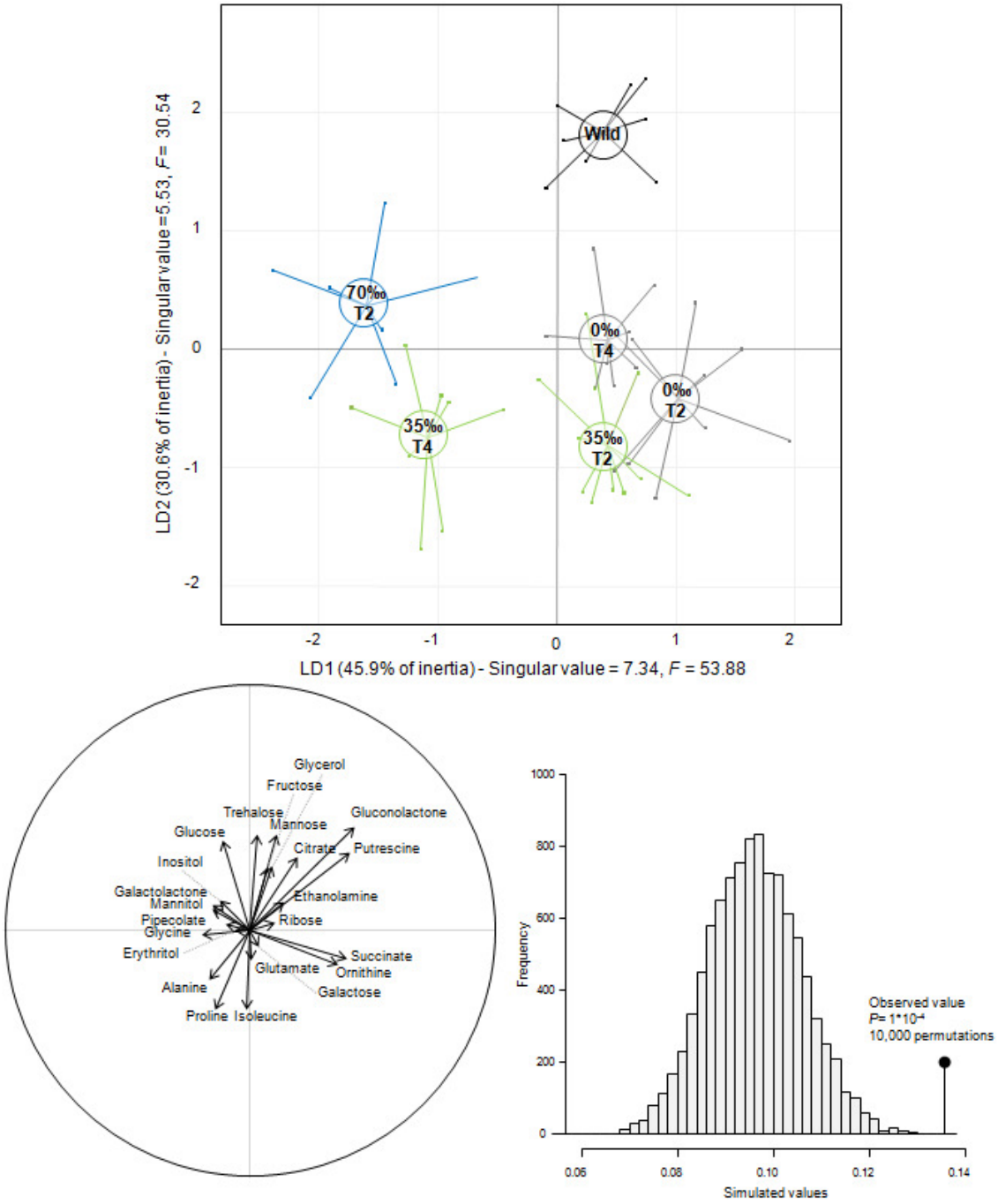

Fig. 5 Sample projection (53 samples) onto the first LDA discriminant plane in individuals maintained at 8 °C. The singular values are the ratio of between-class and within-class inertias. The lines link the samples (three individuals per sample) to the centroid of their class. Black= wild ground beetles; grey= null salinity (0‰); green= medium salinity (35‰); blue= high salinity (70‰). T0= wild ground beetles, T2= two-week exposed ground beetles, and T4= four-week exposed ground beetles. The correlations circle depicts the normed relation (from −1 to 1) between each compound and linear discriminant axes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

4. DISCUSSION

4.1. Impaired survival and body water balance disruption at high salinities

In the present study, we found that food deprivation did not result in any mortality after 4 weeks of experiment, which is consistent with the Lt_{50} of approximately 60 days found in a previous study (Laparie *et al.*, 2012). The combination of food and water deprivation highly significantly altered the survivorship of *M. soledadinus* adults, which dropped to about 14 days at 4 °C and 8 days at 8 °C (data not shown for 8 °C). Intermediate mortalities were found under saline conditions= survivorship remained high to very high in the 4 °C-exposed ground beetles maintained at extreme (70‰) and moderate (35‰) saline conditions, which additionally suggests that the ground beetles drunk salted water from moist sand. There are few available studies that examined the effects of salinity on a terrestrial invertebrate, and the few available ones showed that inland arthropods quickly die when exposed to extreme saline conditions (Pétillon *et al.* 2011). Some of the rare survival data available for terrestrial arthropods inhabiting saline locations indicate survival durations similar to those obtained in the present study (see Article VII) in the dominant ground-dwelling predator in salt marshes, the wolf spider *Arctosa fulvolineata*. In the present study, adults of *M. soledadinus* were maintained in small closed vials, which prevented the access to a source of fresh water that would have offset the environmental salinity. It is thus likely that we significantly underestimated the survival ability of this 6 mm long ground beetle when it is exposed to field conditions.

Thermal conditions had a slight yet additional adverse effect on survival duration. Overall insect activity is driven by environmental temperature, and even a 1 °C rise in environmental temperature strongly increased *M. soledadinus* adult metabolic rate (Lalouette *et al.*, 2012; Todd, 1997). Thus, any increase of the temperature increases the metabolic rate and the amount of salt ingested by the ground beetles (by gas exchange and drinking from moist sand), and consequently heightens the negative effects of salinity. The maintenance of an adequate body water balance represents a prominent problem in insects exposed to hypertonic environments, as they are prone to dehydration. Under saline conditions, insect body water is usually lost during respiratory transpiration and passive body water diffusion occurs from oral and anal

openings (Witteveen *et al.*, 1987). In addition, the deleterious salinity effects on biological and physiological functions likely occurred when the ground beetles drank interstitial saline water. Consistent with this idea, metabolic disruption occurred more quickly in adult *M. soledadinus* exposed at 70‰, likely because the amount of ingested salt was twice higher for the same amount of water drunk than at 35‰. Adults of *M. soledadinus* were able to maintain their body water balance for up to two weeks when they were exposed at medium salinity (35‰), and it is likely that the increased free amino acid pool assisted body water regulation by increasing osmolality. The accumulation of compatible solutes represents a typical plastic response of halotolerant and halophilic arthropods adapted to environmentally saline varied conditions (Benoit, 2010; Martins & Santos, 1995). Over the different classes of metabolites, amino acids represent abundant low molecular organic substances in insects' hemolymph (Renault *et al.*, 2006; Storey *et al.*, 1981), and accumulation of these compounds usually accompanies osmoregulatory adjustments under saline conditions (Edwards, 1982). In the present study, the osmo-induced accumulation of free amino acids, from *ca.* 100 to *ca.* 150 nmol.mg^{-1} dry mass at 0 and 35‰, respectively (at the same time, dry mass was *ca.* 3.6 and 3.5 mg, respectively), may have contributed to increase osmolality of the body fluids, limiting the loss of body water (Hoeger & Abe, 2004). Whether this accumulation in the body water was compartmented among the different body parts or not (see Bishop *et al.*, 1994) will have to be explored in further studies.

A progressive decrease of the body water content and a strong accumulation of amino acids have been observed from the beginning of the experiment onwards in the adults of *M. soledadinus* confined at 70‰. Again, both physiological changes likely contributed to increase the osmotic concentration of the body fluids, as demonstrated in the Antarctic midge, *Belgica antarctica* (Elnitsky *et al.*, 2009). However, the low desiccation tolerance of adult *M. soledadinus* (this study, Todd & Block, 1997) suggests that the disruption of water balance resulting from hyper saline substrate have impaired the survival of the ground beetles. The level of mortality of the ground beetles exposed at 70‰ suggests that they were not able to counterbalance the negative effects of salinity, despite a large accumulation of osmolytes. Similarly, when the ground beetles were exposed at medium salinity for more than two weeks, body

water contents dropped to similar levels as those at 70‰. It remains uncertain whether this decrease resulted from an uncontrolled effect of the salt stress or from controlled body water lost to adjust hemolymph osmolality. Altogether, our data suggest that exposures longer than two weeks at 35‰ progressively became lethal. In nature, it is likely that *M. soledadinus* adults temporarily saunter away from decaying algae and sea wrecks, and gain freshwater from puddles to counterbalance the negative effects of substrate salinity of the intertidal zones.

4.2. Reduction of the energetic metabolism as a result of food deprivation

We found slight but significant signs of energetic metabolism reduction over the course of the experiments, with mannose, fructose, glucose, and citric acid levels being reduced in *M. soledadinus* in the three salinity conditions. The overall reduction of sugar concentrations that occurred in all treatments over the course of the experiment has already been described as one of the metabolic characteristics of starving adult *M. soledadinus* (Laparie *et al.*, 2012), but also from other beetle species (Auerswald & Gäde 2000). The levels of several intermediate metabolites of glycolytic and energy producing pathways are usually decreased during starvation. Reductions in sugar concentration, and more notably monosaccharide and monosaccharide-P pools, which propagate to acid compounds from the tricarboxylic acid cycle (TCA), are common adaptations to salt stress in plant species (Sanchez *et al.*, 2008). Even if monitoring respiration activity would have been necessary to ensure a reduction in catabolic rate, decreased amounts of circulating hexoses and citric acid were consistent with the presumption of a slowdown in energetic metabolism. As this predatory ground beetle relied on fatty body stores (Laparie *et al.*, 2012) after two- and four-week exposures to the experimental conditions, it is likely that triglyceride and glycogen stores were partly depleted.

4.3. Osmolytes were accumulated in response to saline exposure

Higher total metabolite concentrations (which notably exhibited similar levels at 4 and 8 °C) sorted the ground beetles from 0 to 70‰, which supports the hypothesis that physiological changes occurred in response to hypersaline conditions. In addition, temperature intensified the influence of hypersaline conditions on metabolic

signatures, and, congruent with the body water content data, similar metabolic signatures were observed at 8 °C in individuals exposed to 70‰ salinity for two weeks, and four weeks at 35‰ salinity. Even if we have no direct evidences that the ground beetles drunk salted water, their increased survival duration compared to those that were food and water restricted suggest that this event indeed occurred. It is thus likely that differences in metabolite concentrations represent a response to the salinity.

Three amino acids likely explain the total increase in osmolyte concentrations among saline treatments. The pooled concentrations of glycine, proline and alanine varied from about 46 nmoles.mg^{-1} dry mass at 0‰ at 4 and 8 °C to about 102 nmoles.mg^{-1} dry mass at 70‰ at both temperatures. Glycine was the most abundant metabolite in the ground beetles exposed to hypersaline conditions. We have no direct evidence of the potential role of glycine in protecting from osmotic stress in adult *M. soledadinus*, but osmo-induced accumulation of this amino acid has already been reported in other invertebrate species (Siebers *et al.*, 1972; Yancey, 2001). Salinity-dependent accumulation of glycine in adult *M. soledadinus* could play a role in osmoregulatory processes [by assisting retention of body water by osmotic forces, Yancey 2005], together with a non-osmotically function [by protecting macromolecules, and more particularly enzymes (Pollard & Wyn Jones, 1979)].

Increased proline amounts is the most common response in salt-exposed plants, and has already been linked to osmotic adjustments at the cellular level when environmental salinity increases (Misra & Gupta, 2005; Larher *et al.*, 2003). Due to its physico-chemical properties, proline catches hydroxyl radicals induced by water deficits (Yancey, 2005). It has also been shown that this amino acid is involved in the protection of cytosolic and cellular enzyme structure during tissue dehydration (Sanchez *et al.*, 2008). Proline may also act as a chemical chaperone (Sanchez *et al.*, 2008), and, even at low amounts, this amino acid can activate the molecular chaperones (Diamant *et al.*, 2001).

Of particular note is alanine accumulation, which characterized a metabolic signature in the ground beetles exposed at 4 and 8 °C during four weeks. Similarly, significant alanine accumulation was reported in salt-exposed Collembola (Witteveen *et al.*, 1987), spiders, polychaete (Hoeger & Abe, 2004), mosquitoes (Patrick & Bradley, 2000), in some plants (Garnett *et al.*, 2002), or algae (Dittami *et al.*, 2011).

Such a salt-induced accumulation therefore appears as a common response among invertebrate taxa, and more particularly in osmotic conformer organisms.

In addition to amino acids, increased salinity and duration of exposure resulted in a progressive osmo-induced accumulation of erythritol and pipecolate, and reduced amounts of gluconolactone. In plants and bacteria, the intracellular level of pipecolate has been closely related to an increase in extracellular osmotic pressure (Moulin *et al.*, 2002); erythritol was also slightly accumulated in cells of fungi (Kogej *et al.*, 2007). In the present work, the amounts of these two osmolytes were very low, and may have contributed little to osmoregulation processes under hypersaline conditions. The continued reduction in gluconolactone levels, which are comprised of multiple water-attracting hydroxyl groups, likely prevented any potential for osmoprotection. Gluconolactone could result from the degradation of gluconolactone-6-phosphate produced *via* the pentose phosphate pathway. The pentose phosphate pathway is typically elicited in insects exposed to harsh environmental conditions, as it gives rise to several compatible solutes (Storey & Storey, 1991; Koštál *et al.*, 2004), and is a major reductant source (NADPH) (Kruger & von Schaewen, 2003).

5. CONCLUSION

The present study revealed that adult *M. soledadinus* can survive exposures to medium salinities without any access to freshwater or food for up to two weeks, explaining their successful colonization of the tide drift line at the Kerguelen Islands. Temperature increased the deleterious effects of salinity, likely by increasing the rate of body water loss of this poorly dehydration tolerant ground beetle. Dependent on their amounts, the osmo-induced accumulated metabolites may have acted as osmoprotectants, served to adjust hemolymph osmolality, and/or contributed to the management of carbon and reductant power. Glycine and proline were the most abundant metabolites, and exhibited osmo-induced accumulations. The resulting increase in total concentration of these potential osmoprotectants was likely to modulate the ground beetles' body water balance on medium saline substrates, thus enhancing their survival ability. However, it is difficult to ensure the exact role played by glycine during saline exposure based on metabolomics, and the causal relationship

remains to be tested in further studies. Gagneul *et al.* (2007) already concluded that not all organic osmolytes have effective osmoregulatory functions to mitigate salinity effects, and some of them, even if accumulated, only play minor role in salinity tolerance. The importance of inorganic osmolytes in osmoregulatory processes has been stressed in several arthropod species (Naidu, 2001; Koh & Wright, 2011), and the accumulation of these osmolytes that may be differentially compartmented within the body (Wright *et al.* 1997) should be addressed in future research.

Acknowledgements

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REFERENCES

Auerswald, L., *Gäde,* G., 2000. Metabolic changes in the African fruit beetle, *Pachnoda sinuata*, during starvation. Journal of Insect Physiology, 46, 343-351.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate - A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B Statistical Methodology, 57, 289-300.

Benoit, J.B., 2010. Water management by dormant Insects= comparisons between dehydration resistance during summer aestivation and winter diapauses. Progress in Molecular and Subcellular Biology, 49, 209-229.

Bishop, S.H., Greenwalt, D.E., Kapper, M.A., Paynter, K.T., Ellis, L.L., 1994. Metabolic regulation of proline, glycine and alanine accumulation as intracellular osmolytes in ribbed mussel gill tissue. Journal of Experimental Zoology, 268, 151-161.

Diamant, S., Eliahu, N., Rosenthal, D., Goloubinoff, P., 2001. Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. The Journal of Biological Chemistry, 276, 39586-39591.

Dittami, S.M., Gravot, A., Renault, D., Goulitquer, S., Eggert, A., Bouchereau, A., Boyen, C., Tonon, T., 2011. Integrative analysis of metabolite and transcript abundance during the short term response to abiotic stress in the brown alga *Ectocarpus siliculosus*. Plant, Cell and Environment, 34, 629-642.

Edwards, H.A., 1982. Free amino acids as regulators of osmotic pressure in aquatic insect larvae. The Journal of Experimental Biology, 101, 153-160.

Elnitsky, M.A., Benoit, J.B., Lopez-Martinez, G., Denlinger, D.L., Lee, R.E. Jr., 2009. Osmoregulation and salinity tolerance in the Antarctic midge, *Belgica antarctica*= seawater exposure confers enhanced tolerance to freezing and dehydration. The Journal of Experimental Biology, 212, 2864-2871.

Ernsting, G., 1993. Observations on life cycle and feeding ecology of two recently introduced predatory beetle species at South Georgia, sub-Antarctic. Polar Biology, 13, 423-428.

Gagneul, D., Ainouche, A., Duhazé, C., Lugan, R., Larher, F.R., Bouchereau, A., 2007. A reassessment of the function of the so-called compatible solutes in the halophytic Plumbaginaceae Limonium latifolium. Plant Physiology, 144, 1598-1611.

Garnett, M.R., Murch, S.J., KrishnaRaj, S., Dixon, M.A., Saxena, P.K., 2002. The rhizoflitration of sodium from hydroponic fluid using scented geraniums. Water, Air, & Soil Pollution, 140, 343-365.

Hoeger, U., Abe, H., 2004. Beta alanine and other amino acids during salinity adaptation of the polychaete *Nereis japonica*. Comparative Biochemistry and Physiology A, 137, 161-171.

Jeannel, R., 1940. Croisière du Bougainville aux iles australes françaises. III. Coléoptères. Mémoires du Musée National d'Histoire Naturelle série A, 14, 63-202.

Kefford, B.J., Papas, P.J., Metzeling, L., Nugegoda, D., 2004. Do laboratory salinity tolerances of freshwater animals correspond with their field salinity? Environmental Pollution, 129, 355-362.

Kirst, G.O., 1990. Salinity tolerance of eukaryotic marine algae. Annual Review of Plant Physiology, 41, 21-53.

Kogej, T., Stein, M., Volkmann, M., Gorbushina, A.A., Galinski, E.A., Gunde-Cimerman, N., 2007. Osmotic adaptation of the halophilic fungus *Hortaea werneckii*= role of osmolytes and melanization. Microbiology, 153, 4261-4273.

Koh, H., Wright, J., 2011. Cation regulation by the terrestrial isopod *Armadillidium vulgare* (Crustacea= Isopoda= Oniscidea) during dehydration in air. Comparative Biochemistry and Physiology A, 159, 134-140.

Koštál, V., Vambera, J., Bastl, J., 2004. On the nature of pre-freeze mortality in insects= water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. The Journal of Experimental Biology, 207, 1509-1521.

Kruger, N.J., von Schaewen, A., 2003. The oxidative pentose phosphate pathway; structure and organization. Current Opinion in Plant Biology, 6, 236-46.

Lalouette, L., Williams, C.M., Cottin, M., Sinclair, B.J., Renault, D., 2012. Thermal biology of the alien ground beetle *Merizodus soledadinus* introduced to the Kerguelen Islands. Polar Biology, 35, 509-517.

Laparie, M., Lebouvier, M., Lalouette, L., Renault, D., 2010. Variation of morphometric traits in populations of an invasive carabid predator (*Merizodus soledadinus*) within a subantarctic island. Biological Invasions, 12, 3405-3417.

Laparie, M., Larvor, V., Frenot, Y., Renault, D., 2012. Starvation resistance and effects of diet on energy reserves in a predatory ground beetle (*Merizodus soledadinus*; Carabidae) invading the Kerguelen Islands. Comparative Biochemistry and Physiology A, 161, 122-129.

Larher, F.R., Aziz, A., Gibon, Y., Trotel-Aziz, P., Sulpice, R., Bouchereau, A., 2003. An assessment of the physiological properties of the so-called compatible solutes using in vitro experiments with leaf discs. Plant Physiology and Biochemistry, 41, 657-666.

Lebouvier, M., Laparie, M., Hullé, M., Marais, A., Cozic, Y., Lalouette, L., Vernon, P., Candresse, T., Frenot, Y., Renault, D., 2011. The significance of the sub-Antarctic Kerguelen Islands to assess the vulnerability of native communities to climate change, alien insect invasions and plant viruses. Biological Invasions, 13, 1195-1208.

Martins, L.O., Santos, H., 1995. Accumulation of mannosylglycerate and di-myo-inositol-phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Applied and Environmental Microbiology, 6, 3299-3303.

Misra, N., Gupta, A.K., 2005. Effect of salt stress on proline metabolism in two high yieldind genotypes of green gram. Plant Science, 169, 331-339.

Moulin, M., Deleu, C., Larher, F.R., Bouchereau, A., 2002. High-performance liquid chromatography determination of pipecolic acid after precolumn ninhydrin derivatization using domestic microwave. Analytical Biochemistry, 308, 320-327.

Naidu, S.G., 2001. Osmoregulation in *Onymacris rugatipennis*, a free-ranging tenebrionid beetle from the Namib Desert. Comparative Biochemistry and Physiology A, 129, 873-885.

Naidu, S.G., 2006. Haemolymph amino acid, sugar and glycerol levels in the Namib Desert tenebrionid *Physadesmia globosa* (Coleoptera= Tenebrionidae) during dehydration and rehydration. European Journal of Entomology, 103, 305-310.

Nicolson, S.W., 1980. Water balance and osmoregulation in *Onymacris plana*, a tenebrionid beetle from the Namib Desert. Journal of Insect Physiology, 26, 315-320.

Ottesen, P.S., 1990. Diel Activity Patterns of Carabidae, Staphylinidae and Perimylopidae (Coleoptera) at South Georgia, Sub-Antarctic. Polar Biology, 10, 515-519.

Patrick, M.L., Bradley, T.J., 2000. Regulation of compatible solute accumulation in larvae of the mosquito *Culex tarsalis*= osmolarity vs salinity. The Journal of Experimental Biology, 203, 831-839.

Pétillon, J., Georges, A., Canard, A., Lefeuvre, J.C., Bakker, J.P., Ysnel, F., 2008. Influence of abiotic factors on spider and ground beetle communities in different salt marsh systems. Basic and Applied Ecology, 9, 743-751.

Pétillon, J., Lambeets, K., Ract-Madoux, B., Vernon, P., Renault, D., 2011. Saline stress tolerance partly matches with habitat preference in ground-living wolf spiders. Physiological Entomology, 36, 165-172.

Pollard, A., Wyn Jones, R.G., 1979. Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta, 144, 291-298

R Development Core Team, 2008. R= A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, ISBN 3-900051-07-0, URL http=//www.R-project.org.
Renault, D., Bouchereau, A., Delettre, Y. R., Hervant, F., Vernon, P., 2006. Changes in free amino acids in *Alphitobius diaperinus* (Coleoptera= Tenebrionidae) during thermal and food stress. Comparative Biochemistry and Physiology A, 143, 279-285.

Renault, D., 2011. Sea water transport and submersion tolerance as dispersal strategies for the invasive ground beetle *Merizodus soledadinus*. Polar Biology, 34, 1591-1595.

Rhodes-Ondi, S.E., Turner, R., 2010. Salinity tolerance and osmotic response of the estuarine hermit crab *Pagarus maclaughlinae* in the Indian river Lagoon, Florida. Estuarine, Coastal and Shelf Science, 86, 189-196.

Roig-Junent, S., Dominguez, M.C., 2001. Diversidad de la familia Carabidae (Coleoptera) en Chile. Revista Chilena de Historia Natural, 74, 549-571.

Rudloe, A., 1979. Locomotor and light responses of larvae of the horseshoe crab, *Limulus polyphemus* (L.). The Biological Bulletin, 157, 494-505.

Sanchez, F.J., Manzanares, M., De Andres, E.F., Tenorio, J.L., Ayerbe, L., 2008. Turgor maintenance, osmotic adjustment and soluble sugar and proline accumulation in 49 pea cultivars in response to water stress. Field Crop Research, 59, 225-235.

Siaussat, D., Laparie, M., Maria, A., Renault D. Molecular responses to salinity, starvation and temperature in a ground *Merizodus soledadinus* invading the Kerguelen Islands. In press, Polar Biology.

Siebers, D., Lucu, C., Sperling, K.R., Eberlein, K., 1972 Kinetics of osmoregulation in the crab Carcinus maenas. Marine Biology, 17, 291-303.

Sowers, A.D., Young, S.P., Grosell, M., Browdy, C.L., Tomasso, J.R., 2006. Hemolymph osmolality and cation concentrations in *Litopenaeus vannamei* during exposure to artificial sea salt or a mixed-ion solution= Relationship to potassium flux. Comparative Biochemistry and Physiology A, 145, 176-180.

Storey, K.B., Baust, J.G., Storey, J.M., 1981. Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. The Journal of Comparative Physiology B, 144, 183-190.

Storey, K.B., Storey, J.M., 1991. Biochemistry of cryoprotectants. In Insects at low temperature (Denlinger, D., Lee, R.E., eds), pp 64–93. New York= Chapman and Hall.

Todd, C.M., 1997. Respiratory metabolism in two species of carabid beetle from the sub-Antarctic island of South Georgia. Polar Biology, 18, 166-171.

Todd, C.M., Block, W., 1997. Responses to desiccation in four Coleopterans from sub-Antarctic South Georgia. Journal of Insect Physiology, 43, 905-913.

Vaidyanathan, H., Sivakumar, P., Chakrabarty, R., Thomas, G., 2003. Scavenging of reactive oxygen species in NaCl-stressed rice (*Oryza sativa* L.) - differential response in salt-tolerant and sensitive varieties. Plant Science, 165, 1411-1418.

Vernon, P., Vannier, G., Tréhen, P., 1998. A comparative approach to the entomological diversity of polar regions. Acta Oecologica, 19, 303-308.

Wang, Y., Mopper, S., Hasenstein, K.H., 2008. Osmolytes in salinity-stressed *Iris hexagona*. Acta Physiologiae Plantarum, 30, 715-721.

Welch, W.J., Brown, C.R., 1996. Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperon, 1, 109-115

Witteveen, J., Verhoef, H.A., Letschert, J.P.W., 1987. Osmotic and ionic regulation in marine littoral Collembola. Journal of Insect Physiology, 1, 59-66.
Wright, J.C., O'Donnell, M.J., Sazgar, S., 1997. Haemolymph osmoregulation and the fate of sodium and chloride during dehydration in terrestrial isopods. Journal of Insect Physiology, 43, 795-807.

Yancey, P.H., 2001. Protein, osmolytes and water stress. American Zoologist, 41, 699-709.

Yancey, P.H., 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. The Journal of Experimental Biology, 208, 2819-2830.

Deciphering the metabolic changes associated with diapause syndrome and cold acclimation in the two-spotted spider mite *Tetranychus urticae* - (Article VI)

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Abstract

Diapause is a common feature in several arthropod species that are subject to unfavorable growing seasons. The range of environmental cues that trigger the onset and termination of diapause, in addition to associated hormonal, biochemical, and molecular changes, have been studied extensively in recent years; however, such information is only available for a few insect species. Diapause and cold hardening usually occur together in overwintering arthropods, and can be characterized by recording changes to the wealth of molecules present in the tissue, hemolymph, or whole body of organisms. Recent technological advances, such as high throughput screening and quantification of metabolites via chromatographic analyses, are able to identify such molecules. In the present work, we examined the survival ability of diapausing and non-diapausing females of the two-spotted spider mite, Tetranychus urticae, in the presence (0 or 5°C) or absence of cold acclimation. Furthermore, we examined the metabolic fingerprints of these specimens via gas chromatography-mass spectrophotometry (GC-MS). Partial Least Square Discriminant Analysis (PLS-DA) of metabolites revealed that major metabolic variations were related to diapause, indicating in a clear cut-off between diapausing and non-diapausing females, regardless of acclimation state. Signs of metabolic depression were evident in diapausing females, with most amino acids and TCA cycle intermediates being significantly reduced. Out of the 40 accurately quantified metabolites, seven metabolites remained elevated or were accumulated in diapausing mites, i.e. cadaverine, gluconolactone, glucose, inositol, maltose, mannitol and sorbitol. The capacity to accumulate winter polyols during cold-acclimation was restricted to diapausing females. We conclude that the induction of increased cold hardiness in this species is associated with the diapause syndrome, rather than being a direct effect of low temperature. Our results provide novel information about biochemical events related to the cold hardening process in the two-spotted spider mite.

***Keywords*=** Amino acid metabolism, Arthropoda, diapause, glucose, glucose metabolism, insects, metabolic analysis, mites

1. INTRODUCTION

Diapause is a common feature in several arthropod species that are subject to seasonally unfavorable growing periods (Danks, 1987; Koštál, 2006). This genetically programmed developmental response is characterized by a significant slowing (or even cessation) of development, increased body reserves, metabolic suppression, and altered behavior (Denlinger, 2002; Furusawa *et al.*, 1982; Koštál *et al.*, 2000). The range of environmental cues that trigger the onset and termination of diapause, as well as the associated hormonal, biochemical, and molecular changes, have been extensively studied over the recent years (Hahn & Denlinger, 2007; Koštál *et al.*, 2000, 2008, 2009; Ragland *et al.*, 2010; Zhang *et al.*, 2011). However, this information is restricted to a few biological species, with further investigations on non-model arthropod being required to build a comprehensive understanding of diapause phenotype.

Diapause is usually associated with an increase in the degree of resistance to a variety of environmental stresses, which maximize a species' chances of survival (Danks, 1987). Even though there is evidence for the independent occurrence of diapause and cold hardiness (Denlinger, 1986; Masaki, 1980; Tanaka, 1997), both processes are usually linked, occurring in combination in overwintering arthropods (Atapour & Moharramipour, 2009; Goto *et al.*, 2001; Khodayari *et al.*, 2012; Slachta *et al.*, 2002). Diapause represents a prerequisite for subsequent cold hardening in certain insect species, while the non-diapausing species usually exhibit a limited ability for cold acclimation (Slatcha *et al.*, 2002). The enhancement of cold hardiness is a multicomponent process, and involves the biosynthesis of some sugars and polyols. The synthesis of these compounds is promoted by diapause (Lee *et al.*, 1987; Koštál & Simek, 1995; Moreau *et al.*, 1981), and is triggered by exposure to low temperatures (Baust, 1982; Lee *et al.*, 1987; Storey, 1982). At the early stages of insect diapause, a decline in the expression of enzymes involved in energetic metabolism has been observed (Koštál *et al.*, 2008), together with increased amounts of key enzymes involved in polyols production, such as glucose-6-P dehydrogenase, aldose reductase, and ketose reductase (Koštál *et al.*, 2006). Then, during diapause development, the amount of accumulated polyols is modulated (Koštál *et al.*, 2006),

with subsequent declines in temperature expected to have further effect on the synthesis of these compounds.

Current developments in analytical techniques facilitate the exploration of the functional properties of biochemical and physiological changes associated with diapause syndrome (Goodacre *et al.*, 2004; Wolschin & Gadau, 2009). Among these innovative technological advances, high throughput screening and quantification of metabolites via chromatographic analyses may be used to show the quantity of a number of molecules present in the tissue, hemolymph, or whole body of organisms exposed to a given set of conditions (Goodacre *et al.*, 2004). Very few studies used metabolomics, which is a young but rapidly growing discipline in the domain of insect stress physiology (Colinet *et al.*, 2012; Koštál *et al.*, 2012; Laparie *et al.*, 2012; Teets *et al.*, 2012), to explore the metabolic fingerprints of diapausing insects (Michaud & Denlinger, 2004). These authors compared the metabolic signatures of diapausing pupae to non-diapausing cold-hardened pupae of the flesh fly Sarcophaga crassipalpis. A large increase in glucose and pyruvate was reported (Michaud & Denlinger, 2004), which, together with a decline of the molecules of the tricarboxylic acid (TCA) cycle, indicates a decline in aerobic metabolism, with glycolysis being diverted to the pentose phosphate pathway to yield polyols (Koštál *et al.*, 2004). Subsequently, signs of metabolic depression were confirmed by metabolomics in diapausing specimens of the aphid parasitoid Praon volucre, with a drastic reduction in the levels of malate, succinate, and fumarate (Colinet *et al.*, 2010, 2012). However, it remains unknown as to whether the metabolic fingerprints of diapause syndrome are ubiquitous across all insect species.

The two-spotted spider mite, Tetranychus urticae Koch (Acari= Tetranychidae), is a well-known plant-feeding pest species worldwide. Tetranychus urticae females overwinter in a state of diapause. This facultative diapause is induced by short-day conditions during the period of juvenile development of females (Veerman, 1985). In the present work, we examined the physiological similarities and specificities of cold hardiness and diapause by comparing the metabolic fingerprints of diapausing and non-diapausing females of T. urticae in the presence and absence of cold acclimation. In addition to the species' pest status, we believe that this chill tolerant mite (Khodayari *et al.*, 2012), for which the complete genome has been

recently published (Grbic *et al.*, 2011), may soon become a biological model for ecophysiological studies. We report the first metabolomics dataset for this species by conducting GC–MS analyses with an uncommon full automated online derivatization process that ensured a complete quality control. We identified the spectrum of metabolites accumulated by cold-hardening in females of T. urticae, and examined whether the same metabolites also accumulated in diapausing and cold acclimated females. We hypothesized that (1) diapausing females are characterized by a higher ability to survive exposures at low temperatures compared to non-diapausing females, (2) non-diapausing females exhibit limited cold hardening capacities, and (3) metabolic depression, which is a characteristic of diapausing specimens, results in a large decrease in the concentrations of metabolites involved in the TCA cycle, distinguishing the metabotypes of diapausing and non-diapausing females.

2. MATERIALS AND METHODS

2.1. Collection and Rearing of Mites

Adults of T. urticae were originally hand-collected during the summer of 2010 from a bean field near the Tarbiat Modares University in Tehran, Iran (35°44′N, 51°10′E). Adults were directly transferred to the laboratory, and reared on beans under controlled conditions (Light/Dark= 16/8 h; Relative humidity= 70%; Temperature= 27±1°C). Then, more than 30 reared females were sampled at random, and were immediately transferred to detached bean leaves (L/D= 16/8 h; RH= 70%; Temperature= 27±1°C). These females were allowed to lay eggs for 24 h, before being removed from the leaves. Detached bean leaves were placed upside down on a layer of wet cotton in Petri dishes (9 cm in diameter). Each day, water was added to the cotton to keep the arena humid. The lids of the Petri dishes had a 3 cm diameter hole covered with fine nylon mesh to allow ventilation. Hundreds of offspring were then reared until the adult stage, at temperatures of 25°C (L/D= 16/8 h), or 20°C (L/D= 8/16 h), to obtain non-diapausing (ND) and diapausing (D) females, respectively. About 2 weeks was required for ND specimens and 4 weeks for D specimens to reach the adult stage. Subsequently, two batches of newly emerged ND and D females were made. ND and D females from the first batch were directly used in survival experiments, and in

metabolic fingerprinting assays. ND and D females from the second batch were randomly transferred to 5°C (NDA5, DA5) or 0°C (NDA0, DA0) for 10 d, at a photoperiod of 12/12 h (L/D) for ND females and 8/16 h (L/D) for D females. In total, there were six experimental conditions= ND, D, NDA5, DA5, NDA0, and DA0 females.

No specific permits were required for the described field studies. The location were the spider mites were sampled are in not privately-owned and not protected in any way. The spider mites represent pest species in Iran, and as such, are not endangered or protected species in any way.

2.2. Survival to Acute Cold Stress

Acclimated (NDA5, NDA0, DA5, and DA0) and non-acclimated (ND and D) females were exposed to subzero temperatures above their supercooling point (SCP) (see (Khodayari *et al.*, 2012) for SCP values of female T. urticae), to assess mortality caused by cold-related factors other than freezing. Each group of females was transferred to small microtubes (0.5 ml), and was then directly cooled from 25°C to −5, −10, −15, and −20°C at 1°C min^{-1}, and kept at these temperatures for 24 h. Four to six replicates were completed for each experimental treatment, with each replicate containing 10 females. The females were subsequently transferred to 25°C (RH= 70%) for recovery. Mortality was determined at 24 h after recovery. Moving specimens, including those that were not able to walk, were considered as alive.

2.3. Effects of Acclimation on the Metabolic Fingerprints of ND and D Mites

Metabolic fingerprinting was conducted in ND, D, NDA5, and DA5 females (4 to 7 replicates per experimental condition, each replicate containing a pool of 100 females). Each sample was weighed (fresh mass) using a Sartorius micro-balance (sensitivity of 0.01 mg), snap-frozen, and stored at −20°C before use in the biochemical assays.

2.3.1. Sample Preparation and Derivatization

The samples were homogenized in 300 µl of ice-cold (−20°C) methanol-chloroform solution (2=1) using a tungsten-bead beating apparatus (RetschTM MM301, Retsch

GmbH, Haan, Germany) at 25 agitations per second for 1.5 min. Then, 200 µl of ice-cold ultrapure water was added to each sample and then vortexed. After centrifugation at 4,000 g for 5 min at 4°C, 300 µl of upper aqueous phase (which contained polar metabolites) were transferred to new chromatographic glass vials. The vials containing the aliquots were vacuum-dried using a Speed Vac Concentrator (MiVac, Genevac Ltd., Ipswitch, England). The dried aliquots were resuspended in 30 µl of 20 mg. L^{-1} methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine, incubated under automatic orbital shaking at 40°C for 90 min. Then, 30 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma, #394866) was added and the derivatization was conducted at 40°C for 45 min under agitation. All the derivatization process was automatized using CTC CombiPal autosampler (GERSTEL GmbH and Co.KG, Mülheim an der Ruhr, Germany) (**Supplementary data 1**). Our innovative and uncommon analytical procedure ensures identical derivatization time and process for all samples, and represents a prerequisite for a total quality control.

2.3.2. GC-MS Analyses

Our GC-MS system consisted of a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). The injector temperature was held at 250°C. The oven temperature ranged from 70 to 170°C at 5°C.min^{-1}, from 170 to 280°C at 7°C.min^{-1}, from 280 to 320°C at 15°C.min^{-1}, and then the oven remained 4 min at 320°C. We used a 30 m fused silica column (TR5 MS, I.D. 25 mm, 95% dimethyl siloxane, 5% Phenyl Polysilphenylene-siloxane) with helium gas as the carrier at a rate of 1 ml.min^{-1}. One microliter of each sample was injected using the split mode (split ratio= 25=1). We completely randomized the injection order of the samples. The temperature of the ion source was set at 250°C and the MS transfer line at 300°C. Detection was achieved using MS detection in electronic impact (EI). In the present work, we used the selective ion monitoring mode (SIM) (electron energy= −70 eV), ensuring a precise annotation of the detected peaks. Then, we searched for the metabolites that were included in our spectral database (58 pure reference compounds, including the internal standard; Table S1). The peaks were accurately annotated using both their mass spectra (two specific ions) and their retention time. Calibration curves were set using samples consisting of

58 pure reference compounds at levels of 1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000, 1500 and 2000 µM. Chromatograms were deconvoluted using XCalibur v2.0.7 software (Thermo Fischer Scientific Inc, Waltham, MA, USA). Metabolite levels were quantified according to their calibration curves.

2.4. Statistical Analyses

Two-way ANOVA with an acceptable significance level of $P<0.05$ was performed for cold tolerance data analysis with SPSS version 16.0 for windows. The correlation between survival and experimental temperature was determined using a binary logistic regression model (38). Among non-structural carbohydrates, amino acids and organic acids, 47 compounds (out of 58 from our library) were identified in female T. urticae, of which 7 were below the quantification limit of the GC-MS (see **Supplementary data 2**). These seven compounds (dopamine, erythritol, galacturonic acid, gentiobiose, saccharose, succinate, and trehalose) were discarded from the multivariate analyses. First, metabolite concentrations, which were expressed as nmoles/mg fresh mass, were log-transformed. Variations in the quantities of total metabolites, total amino acids, and individual metabolites were then examined among the four experimental conditions (D, ND, DA5, NDA5) using an ANOVA with a Tukey post-hoc procedure. Second, the cube root transformation of the metabolite quantities was conducted, and the effects of diapause and cold acclimation on the metabolic signatures were investigated using Partial-Least Squares Discriminant Analysis (PLS-DA). Multiple correlation coefficient (R^2) and cross-validated R^2(Q^2) were used to confirm the predictive power of the fitted model, and the statistical significance of the PLS-DA was also assessed with permutation tests (1000 permutations, $P<0.001$). Variable Importance in Projection (VIP) scores, which are the weighted sums of squares of the PLS loadings, were obtained from the PLS-DA. For the metabolic pathway analysis, the name of the compounds that exhibited significant variations were used, and the significance of the pathway name was assessed with the Holm-Bonferroni method. A heatmap was constructed using Ward's method as the clustering method and the Pearson correlation as the distance measure. All analyses were conducted using the statistical software of 'R 2.13.0' (R development Core Team, 2008) and MetaboAnalyst (http=//www.Metaboanalyst.ca; Xia *et al.*, 2012).

3. RESULTS

3.1. Survival of Female Tetranychus urticae to Acute Cold Stress

The mortality rates of females exposed to −5, −10, −15, and −20°C for 24 h are shown in **Fig 1**. Mortality levels significantly differed among groups (P<0.05)= DA5 females exhibited the lowest mortality of all experimental conditions. The estimates of temperatures at which 50% of mites died (LT_{50}) are shown in **Fig. 2**. Overall, D females were characterized by a lower LT_{50} compared to ND females, with DA5 females exhibiting the highest cold tolerance (i.e. the lowest LT_{50}).

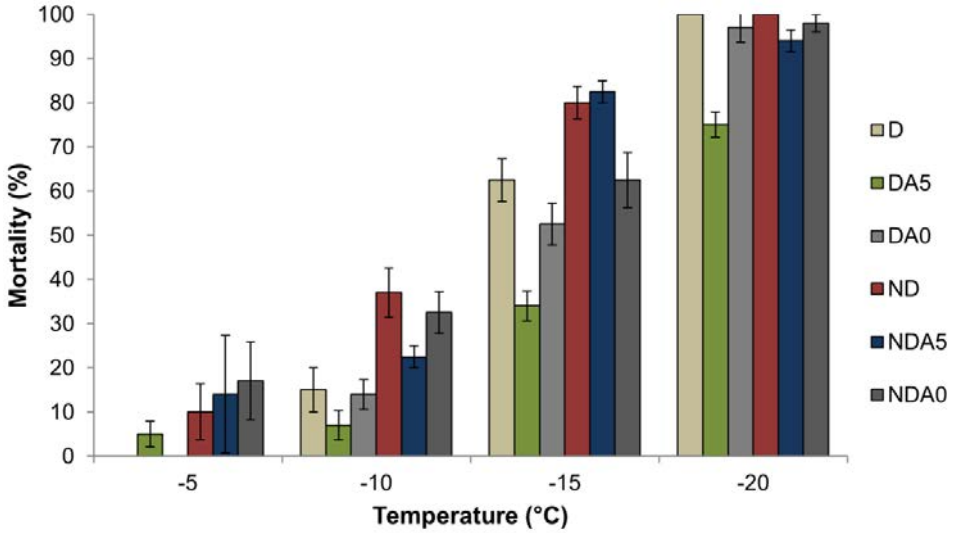

Fig.1 Mortality (mean ± S.E.) was examined in non-diapausing (ND) and diapausing (D) *Tetranychus urticae*females after cold exposures at −5, −10, −15, and −20°C. Non-acclimated mites= ND, D; Mites acclimated at 5°C= NDA5, DA5; Mites acclimated at 0°C= NDA0, DA0.

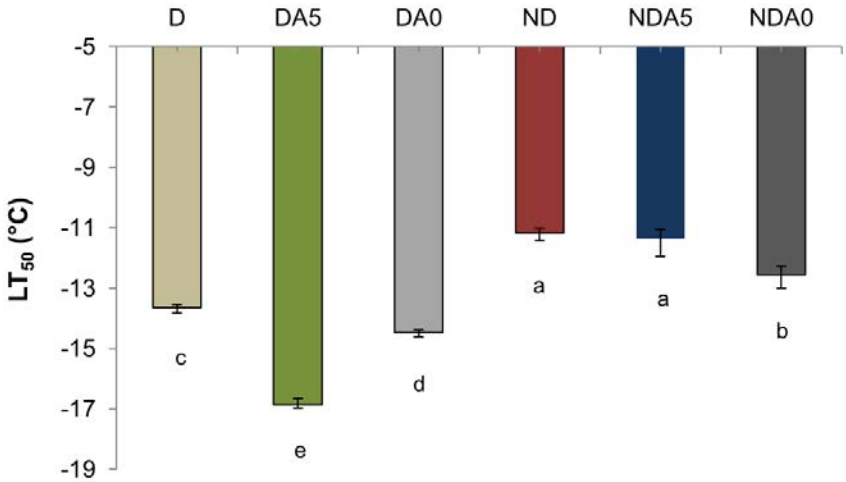

Fig.2 Lethal temperature for 50% percent of the population (LT_{50}) (± confidence intervals) were determined in non-acclimated mites= ND, D; Mites acclimated at 5°C= NDA5, DA5; Mites acclimated at 0°C= NDA0, DA0. Bars with different letters are significantly different (P<0.05).

3.2. Total Amount of Metabolite in Female Tetranychus urticae

A total of 47 metabolites were identified, but only 40 were accurately quantified (**Supplementary data 2**). Of the 40 metabolites, 34 varied significantly among the 4 experimental groups (ND, NDA5, D, DA5; the six non-varying metabolites were= ala, galactose, galactitol, glycerate, triethanolamine, and quinate). Total metabolite and amino acid concentrations significantly differed among the four experimental treatments (Total metabolites= F_3 = 6.12, P<0.01, **Fig. 3A**; Total amino acids= F_3 = 14.19, P<0.001, **Fig. 3B**). The Tukey post-hoc test (α = 0.05) revealed that ND females had the highest total concentrations (70.9±9.4 nmol.mg^{-1} of fresh mass and 165.5±19.4 nmol.mg^{-1} of fresh mass on average ± SE, for total amino acids and total metabolites, respectively), whereas there was no significant difference for the other acclimated females of T. urticae, which had lower concentrations of these compounds (ca. 27 and 100 nmol.mg^{-1} of fresh mass for total amino acids and total metabolites, respectively) (**Fig. 3A-B**).

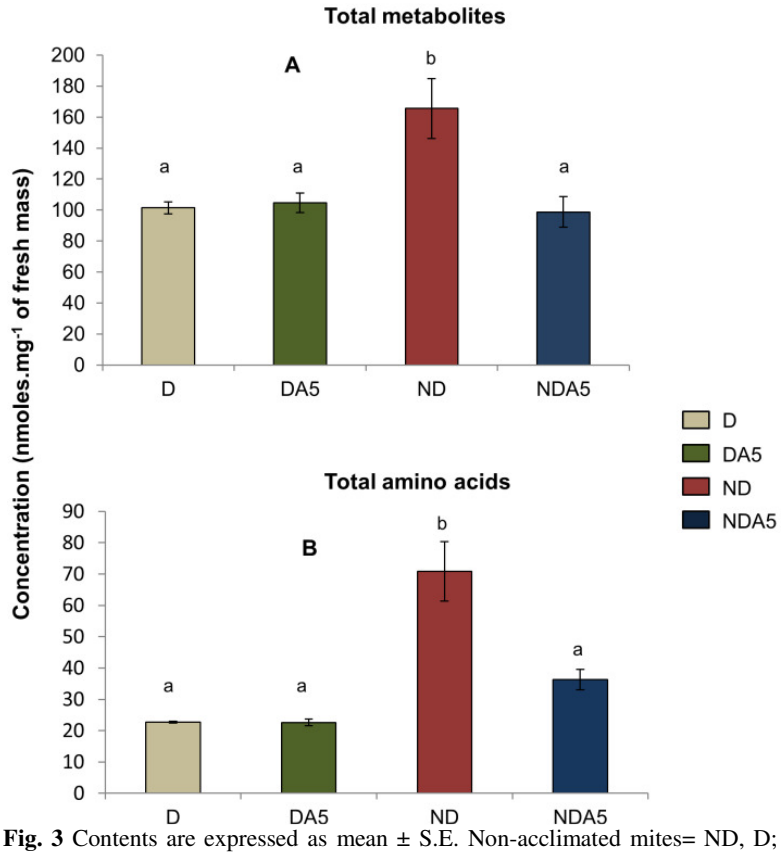

Fig. 3 Contents are expressed as mean ± S.E. Non-acclimated mites= ND, D; Mites acclimated at 5°C= NDA5, DA5. Bars with different letters are significantly different (*P*<0.05).

3.3. Effects of Acclimation on the Metabolic Fingerprints of ND and D Mites

Class separation by the 34 discriminant variables was investigated in a PLS-DA (**Fig. 4A**). The first (LD1) and second (LD2) Linear Discriminant axes accounted for 67.0 and 16.4% of total inertia, respectively. A clear separation among groups was found on LD1, which opposed ND specimens to D ones, regardless of acclimation treatment (**Fig. 4A**). The VIP scores showed that separation on LD1 was mainly due to malate and glucose (**Fig. 4B**). D and DA5 females were characterized by high amounts of cadaverine, gluconolactone, inositol, glucose, maltose, mannitol and sorbitol, and lower amounts of almost all remaining compounds (**Figs. 4B and 5**). Based on the seven listed metabolites, metabolite pathway analysis revealed that only the galactose pathway was significantly enriched (Holm-Bonferroni test, $P<0.05$) in D specimens.

D and DA5 groups continued to overlap on the 2D-projection of the PLS-DA, and ND and NDA5 females were secondarily separated on LD2 (**Fig. 4A**). A heatmap diagram showed that the metabolic signatures of D and DA5 females were similar, with the levels of a number of metabolites being clearly down regulated (**Fig. 6**). Distinct metabolic signatures were more apparent between ND and NDA5, as depicted by the dendrogram (**Fig. 6**).

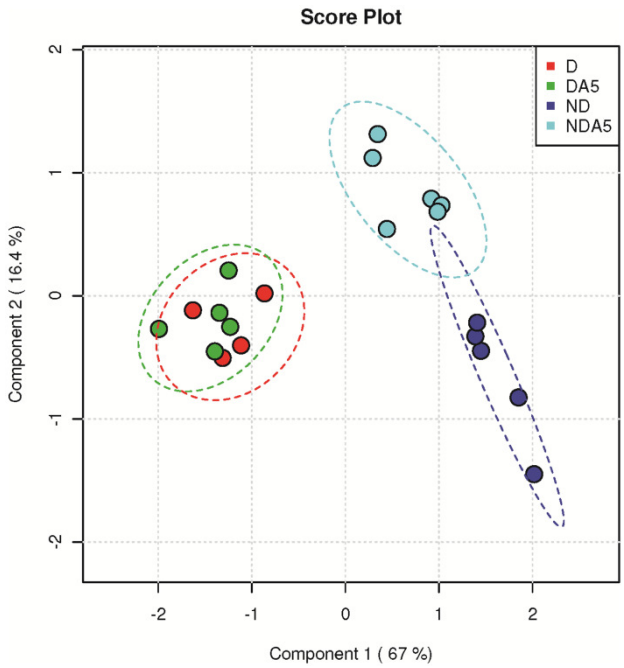

Fig. 4 A= sample projection (4–7 samples) onto the first PLS-DA discriminant plane of non-diapausing (ND) and diapausing (D) *Tetranychus urticae* females, that were non-acclimated (ND, D) and acclimated at 5°C (NDA5, DA5). B= the variable importance plot shows the metabolites that contributed the most to the first axis (based on VIP scores).

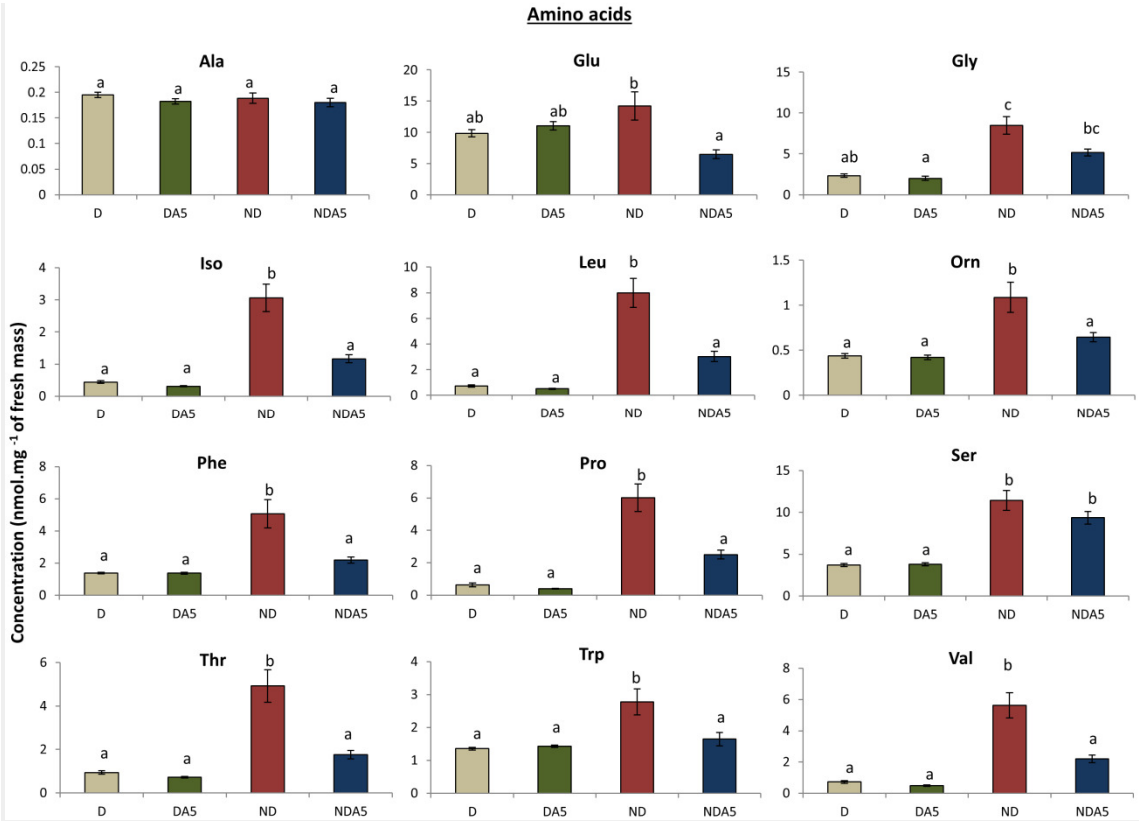

Fig. 5 Metabolite content is expressed as mean ± S.E. Non-acclimated mites= ND, D; Mites acclimated at 5°C= NDA5, DA5. Bars with different letters are significantly different (P<0.05).

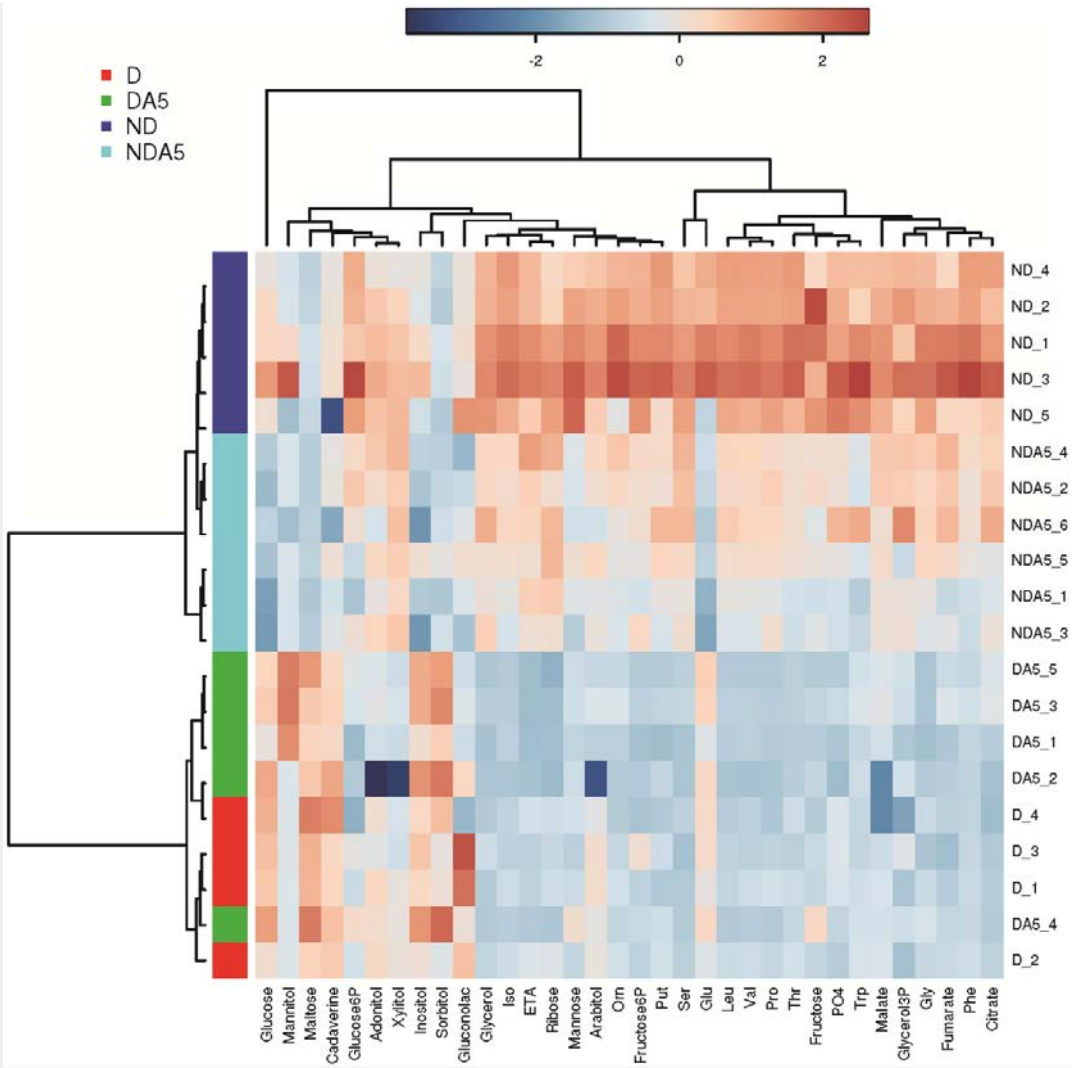

Fig. 6 The colors represent the cube root changes of each metabolite relative to the mean control level. Individual samples (horizontal axis) and compounds (vertical axis) are separated using hierarchical clustering (Ward's algorithm), with the dendrogram being scaled to represent the distance between each branch (distance measure= Pearson's correlation). The clusters containing D and DA5 females are highlighted red and green, respectively. The clusters containing ND and NDA5 females are highlighted in dark and light blue, respectively.

4. DISCUSSION

4.1. Diapause Enhances the Level of Cold Hardiness of Tetranychus urticae Females

In the present study, the physiological basis of cold tolerance in female T. urticae spider mites was examined. Congruent with our previous study (Khodayari *et al.*, 2012), diapause significantly increased the level of cold hardiness in this species= DA5 females exhibited the highest survival ability of all experimental conditions. As

the ND and D females were reared at slightly different temperatures (25 versus 20°C) before being cold acclimated, this procedure may have slightly increased the differences in thermal tolerance between ND and D mites. Supporting our hypotheses, acclimation had a slight beneficial effect on ND specimens, with LT_{50} ranging fromca. −11 to ca. −12°C, depending on the acclimation treatment. Conversely, LT_{50} ranged from −13.5 to −17°C in D mites. The beneficial effects of acclimation on the survival of T. urticae ND females were more visible when the duration of cold-exposure was significantly shorter (Khodayari *et al.*, 2012). By increasing the duration of pre exposure to acute thermal conditions before assessing the survival ability of mites, chill injuries may have accumulated to such an extent that we exceeded the point at which the effects of acclimation could be significantly visible in ND mites. In contrast the opposite results were obtained for diapausing mites, with survival ability being significantly improved when females were acclimated. Diapause is usually associated with enhanced cold tolerance in insects (Pullin, 1996); however, diapausing specimens usually become more cold-hardy when subject to cold exposure (Slatcha *et al.*, 2002). In support of the published literature, our results indicate that additional physiological changes probably occurred during cold acclimation in D females, and better protected mites by enhancing their level of cold tolerance.

4.2. Metabolic Signatures Depicted the Depressed Metabolism of Diapausing Mites

As large numbers of specimens were required for each biological replicate in the metabolic fingerprinting analyses, we focused on NDA5 and DA5 specimens. Data about the cold hardiness of the two-spotted spider mite remain limited, with the current study providing the first report about the metabolome of this species. Hence, our preliminary research about the plastic rearrangement of the metabolic networks of D and ND females provides baseline information regarding the biochemical adjustments that occur during diapause and acclimation in this species.

Distinct metabolic signatures were observed among mites exposed to distinct experimental conditions, indicating that the metabolic networks were rearranged to maintain metabolic homeostasis and performance of the organisms (Szymanski *et al.*, 2009). Diapause had the strongest effect on the metabolic signature of female T.

urticae. In these specimens, we observed a down-regulation in the quantity of several of the measured compounds, including most amino acids and some sugars, supporting that reported for the aphid parasitoid Praon volucre (Colinet *et al.*, 2012). Several of these metabolic changes arise in relation to diapause syndrome, which is characterized by strong metabolic depression and developmental arrest (Colinet *et al.*, 2012. Michaud & Denlinger, 2007). The entry of D females into a hypometabolic state, which was depicted by lower amounts of TCA cycle intermediates compared to ND females, reduces the energetic demand at the level of the whole organism, and has been considered as an adaptive energy-saving strategy (Evans, 1981; Lee, 1980). Developmental arrest also contributed towards explaining the significant reduction of most amino acids, as these biochemical compounds represent the elementary components of many biological structures, including cuticle formation in arthropods and vitellogenesis in mature females.

Most amino acids were down regulated after diapause induction, with only the levels of alanine and glutamate amino acids remaining high in D females of T. urticae. It is not clear to what extent these amino acids may be involved in the response mosaics that assist the winter survival strategy of two-spotted spider mite females; however, some authors have reported their importance in diapausing and cold acclimated insects (Goto *et al.*, 1997; Morgan & Chippendale, 1983; Storey & Storey, 1990). For instance, a lower acclimation temperature, together with diapause, increased the amount of alanine in Mamestra brassicae L. (Goto *et al.*, 2001), which may have occurred as a result of the low aerobic conditions experienced by the insects during diapause, rather than temperature or diapause. Furthermore, an increase in alanine and glutamate was reported in the fat body of Osmoderma eremicola (Knoch) larvae during freezing to −8°C for 96 h (Storey *et al.*, 1993). Proline, which is a well-known energy source and cryoprotectant in some insects (Okasaki & Yamashita, 1981), decreased after diapause induction and cold acclimation. It seems that this amino acid is used as an energy source to fuel aerobic metabolism, and is converted to alanine during diapause. Proline levels were also reduced during diapause in the embryo of Bombyx mori (L.) (Osanai & Yonezawa, 1986), and in chilled diapausing larvae of the grass stem borer, Enosima leucotaeniella (Ragonot) (Goto *et al.*, 1997).

While the metabolic fingerprints of D and DA5 females overlapped, the metabolic signatures of ND and NDA5 females were clearly distinguished. Combined with the survival data, our results demonstrated that diapause is a prerequisite for enhanced cold tolerance in T. urticae females. No polyols were accumulated in NDA5 females, and the reduction of the whole metabolome, including glucose and TCA cycle intermediates, may have only indicated a general decrease in temperature-dependent aerobic activities.

4.3. The Galactose Pathway was Boosted for the Production of Polyols in Diapausing Females

While the levels of most metabolites were reduced in D females, the concentrations of glucose remained stable, regardless of experimental condition. Glucose represented 50% of the total metabolite pool in D mites versus 30% in ND individuals. Furthermore, the high levels of glucose, maltose, inositol, mannitol, sorbitol, and gluconolactone indicated that the pentose phosphate pathway was boosted in D females. As far as we know, earthworms and amphibians are the only animal groups that utilize glucose as their primary cryoprotectant (Costanzo *et al.*, 1993 ; Holmstrup *et al.*, 2007), although this osmolyte may have some potential disadvantages (Calderon *et al.*, 2009). In the fruit fly Drosophila melanogaster, even though glucose was hypothesized to have a role in the rapid cold hardening response (Overgaard *et al.*, 2007), the quantity of glucose was not related to the level of basal thermo-tolerance of flies (MasMillan *et al.*, 2009).

Elevated quantities of glucose are a common feature in diapausing arthropods (Colinet *et al.*, 2012; Koštál *et al.*, 2007; Michaud & Denlinger, 2007; Soudi & Moharramipour, 2012; this study). Consistent with the high levels of glucose in D mites, we also found an accumulation of gluconolactone. Gluconolactone represents an oxidized derivative of glucose, and might serve as an important scavenger of free radicals in diapausing mites. In addition, gluconolactone is the precursor of gluconolactone-6-phosphate in the pentose phosphate pathway, which is typically elicited in insects exposed to harsh environmental conditions (Koštál *et al.*, 2004). The pentose phosphate pathway is a major reductant source in the form of NADPH (Kruger & von Schaewen, 2003), which may be re oxidized for the synthesis

of sorbitol. A 20-fold increase in the activity of aldose reductase, the NADP(H)-dependent enzyme catalyzing the transformation of glucose to sorbitol (Yaginuma & Yamashita, 1979), has already been demonstrated in diapausing specimens of the red firebug Pyrrhocoris apterus (Koštál *et al.*, 2004). In the present study, sorbitol increased two-fold in D T. urticae females, with a 4-fold increase being found in DA5 individuals. Sorbitol is a well-known cryoprotectant in insects (Salvucci, 2000; Wang & Kan, 2005), which is assumed to contribute to survival at low temperatures in a similar manner to glycerol.

Other than sorbitol, mannitol, and, to a lesser extent, inositol, were specifically accumulated in diapausing specimens, supporting that found in the aphid parasitoid P. volucre (Colinet *et al.*, 2012). Diapausing and cold-acclimated arthropods are usually characterized by a partial dehydration, and thus, slightly higher concentrations of these compounds may have been reported in female T. urticae if metabolite concentrations had been expressed per mg dry mass. Accumulations of mannitol (Hendrix & Salvucci, 1998 ; Koštál *et al.*, 2001; Saeidi *et al.*, in press) and inositol (Koštál *et al.*, 2007; Kruger & von Schaewen, 2003; Soudi & Moharramipour, 2012) have also been reported in some other arthropod species. The synthesis of polyols is assumed to coincide with diapause in many insect species (Koštál *et al.*, 2004; Hokova & Hodek, 2004), with these compounds playing a significant role in overwintering success. While polyols had no significant effect on supercooling ability in diapausing P. apterus, polyol (sorbitol and ribitol) accumulation resulted in a 10-fold increase in survival duration, possibly because of the preferential exclusion of solutes from macromolecules (Koštál *et al.*, 2001; Hokova & Hodek, 2004).

Interestingly, glycerol, which is the most commonly known polyol that is produced in cold-hardy insects (Salt, 1961; Storey & Storey, 1988), was consistently low in T. urticae. The highest amounts of glycerol were found in ND females, which decreased after cold acclimation and diapause induction. A similar result was reported for Pieris brassicae (L) (Pullin & Bale, 1989). Hence, it is thus unlikely that glycerol played a role in the thermotolerance of the two-spotted spider mite in the current study.

5. CONCLUSION

To conclude, our study confirmed that the induction of increased cold hardiness is associated with diapause in T. urticae females. Non-diapausing females were characterized by a limited ability to become cold acclimated, which confirmed that diapause is a prerequisite for enhanced cold tolerance in species overwintering in a diapause state. Our metabolic fingerprints were consistent with investigations on enzymatic activities conducted in a previous study (Koštál *et al.*, 2004). While the levels of most compounds were down regulated in D females, glucose levels remained elevated, together with the high concentrations of gluconolactone, which may be involved in the formation of reducing equivalents that may be involved in the production and accumulation of polyols (i.e., sorbitol, mannitol, and inositol).

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REFERENCES

Danks HV (1987) Insect dormancy= An ecological perspective. Biological Survey of Canada (Terrestrial Arthorpods), Monograph series No. 1, Ottawa.

Koštál V (2006) Eco-physiological phases of insect diapause. J Insect Physiol 52=113–127.

Furusawa T, Shikata M, Yamashita O (1982) Temperature dependent sorbitol utilization in diapause eggs of the silkworm, Bombyx mori. J Comp Physiol B 147= 21–26.

Koštál V, Shimada K, Hayakawa Y (2000) Induction and development of winter larval diapause in a drosophilid fly, Chymomyza costata. J Insect Physiol 46= 417–428.

Denlinger DL (2002) Regulation of diapause. Annu Rev Entomol 47= 93–122.

Hahn DA, Denlinger DL (2011) Energetics of Insect Diapause. Annu Rev Entomol 56= 103–121.

Koštál V, Noguchi H, Shimada K, Hayakawa Y (2000) Circadian component influences the photoperiodic induction of diapause in a drosophilid fly, Chymomyza costata. J Insect Physiol 46= 887–896.

Koštál V, Tollarova M, Dolezel D (2008) Dynamism in physiology and gene transcription during reproductive diapause in a heteropteran bug, Pyrrhocoris apterus. J Insect Physiol 54= 77–88.

Koštál V, Simunkova P, Kobelkova A, Shimada K (2009) Cell cycle arrest as a hallmark of insect diapause= Changes in gene transcription during diapause induction in the drosophilid fly, Chymomyza costata. Insect Biochem Mol Biol 39= 875–883.

Hahn DA, Denlinger DL (2007) Meeting the energetic demands of insect diapause= nutrient storage and utilization. J Insect Physiol 53= 760–773.

Ragland GJ, Denlinger DL, Hahn DA (2010) Mechanisms of suspended animation are revealed by transcript profiling of diapause in the flesh fly. Proc Natl Acad Sci USA 107= 14909–14914.

Zhang Q, Nachmanc RJ, Kaczmarekc K, Zabrockic J, Denlinger DL (2011) Disruption of insect diapause using agonists and an antagonist of diapause hormone. Proc Natl Acad Sci USA 108= 16922–16926.

Denlinger DL (1986) Dormancy in tropical insects. Annu Rev Entomol 31= 239–264.

Masaki S (1980) Summer diapause. Annu Rev Entomol 25= 1–25.

Tanaka K (1997) Evolutionary relationship between diapause and cold hardiness in the house spider, Achaearanea tepidariorum (Araneae= Theridiidae). J Insect Physiol 43= 271–274.

Goto M, Sekine Y, Outa H, Hujikura M, Suzuki K (2001) Relationships between cold hardiness and diapause, and between glycerol and free amino acid contents in overwintering larvae of the oriental corn borer, Ostrinia furnacalis. J Insect Physiol 47= 157–165.

Slachta M, Vambera J, Zahradnickova H, Koštál V (2002) Entering diapause is a prerequisite for successful cold-acclimation in adult Graphosoma lineatum (Heteroptera= Pentatomidae). J Insect Physiol 48= 1031–1039.

Atapour M, Moharramipour S (2009) Changes of cold hardiness, supercooling capacity and major cryoprotectants in overwintering larvae of Chilo suppressalis (Lepidoptera= Pyralidae). Environ Entomol 38= 260–265.

Khodayari S, Moharramipour S, Kamali K, Jalali Javaran M, Renault D (2012) Effects of acclimation and diapause on the thermal tolerance of the two-spotted spider mite Tetranychus urticae. J Therm Biol 37= 419–423.

Moreau R, Oliver D, Gourdoux L, Dutrieu J (1981) Carbohydrate metabolism in Pieris brassicae L. (Lepidoptera)= variations during normal and diapausing development. Comp Biochem Physiol B 68= 95–99.

Lee RE Jr, Chen CP, Denlinger DL (1987) A rapid cold-hardening process in insects. Science 238= 1415–1417.

Koštál V, Simek P (1995) Dynamics of cold hardiness, supercooling and cryoprotectants in diapausing and non diapausing pupae of the cabbage root fly, Delia radicum L. J Insect Physiol 41= 627–634.

Baust JG (1982) Environmental triggers to cold hardening. Comp Biochem Physiol A 73= 563–570.

Storey KB (1982) Phosphofructokinase from the overwintering gall fly larva, Eurosta solidaginis= control of cryoprotectant polyol synthesis. Insect Biochem 12= 501–505.

Lee RE Jr, Chen CP, Meacham MH, Denlinger DL (1987) Ontogenetic patterns of cold-hardiness and glycerol production in Sarcophaga crassipalpis. J Insect Physiol 33= 587–592.

Koštál V, Tollarova M, Sula J (2004) Adjustments of the enzymatic complement for polyol biosynthesis and accumulation in diapausing cold-acclimated adults of Pyrrhocoris apterus. J Insect Physiol 50= 303–313.

Wolschin F, Gadau J (2009) Deciphering proteomic signatures of early diapause in Nasonia. PLoS One 4= e6394.

Colinet H, Renault D, Charoy-Guevel B, Com E (2012) Metabolic and proteomic profiling of diapause in the aphid parasitoid Praon volucre. PLoS One 7= e32606.

Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB (2004) Metabolomics by numbers= acquiring and understanding global metabolite data. Trends Biotechnol 22= 245–252.

Colinet H, Larvor V, Laparie M, Renault D (2012) Exploring the plastic response to cold acclimation through metabolomics. Funct Ecol 26= 711–722.

Koštál V, Simek P, Zahradnickova H, Cimlova J, Stetina T (2012) Conversion of the chill susceptible fruit fly larva (Drosophila melanogaster) to a freeze tolerant organism. Proc Natl Acad Sci USA 109= 3270–3274.

Laparie M, Bical R, Larvor V, Vernon P, Frenot Y, et al. (2012) Habitat phenotyping of two sub-Antarctic flies by metabolic fingerprinting= evidence for a species outside its home? Comp Biochem Physiol A 162= 406–412.

Teets NM, Peyton JT, Ragland GJ, Colinet H, Renault D, et al. (2012) Uncovering the molecular mechanisms of rapid cold-hardening and cold shock recovery in a temperate flesh fly using a combined transcriptomic and metabolomic approach. Physiol Genomics 44= 764–777.

Michaud MR, Denlinger DL (2007) Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (Sarcophaga crassipalpis)= a metabolomic comparison. J Comp Physiol B 177= 753–763.

Colinet H, Muratori F, Hance T (2010) Cold-induced expression of diapause in Praon volucre= fitness cost and morpho-physiological characterization. Physiol Entomol 35= 301–307.

Veerman A (1985) Diapause. In= Helle W, Sabelis MW, editors. Spider Mites, their Biology, Natural Enemies and Control. Amsterdam. 279–316.

Grbic M, Van Leeuwen T, Clark RM, Rombauts S, Rouze´ P, *et al.* (2011) The genome of Tetranychus urticae reveals herbivorous pest adaptations. Nature 479=
487–492.

Berkvens N, Bale JS, Berkvens D, Tirry L, De Clercq P (2010) Cold tolerance of the harlequin ladybird Harmonia axyridis in Europe. J Insect Physiol 56= 438–444.

R Development Core Team (2008) R= A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, ISBN 3– 900051–07–0, URL http=//www.R-project.org. Accessed 2012 December 15.

Xia J, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS (2012) MetaboAnalyst 2.0 - a comprehensive server for metabolomic data analysis. Nucl Acids Res doi=10.1093/nar/gks374.

Pullin AS (1996) Physiological relationships between insect diapause and cold tolerance= Coevolution or coincidence? Eur J Entomol 93= 121–129.

Szymanski J, Jozefczuk S, Nikoloski Z, Selbig J, Nikiforova V, et al. (2009) Stability of Metabolic Correlations under Changing Environmental Conditions in Escherichia coli – A Systems Approach. PLoS One 4= e7441.

Lee RE Jr (1980) Physiological adaptations of coccinellidae to supranivean and subnivean hibernacula. J Insect Physiol 26= 135–138.

Evans DE (1981) Thermal acclimation in several species of stored-grain beetles. Aust J Zool 29= 483–492.

Morgan TD, Chippendale GM (1983) Free amino acids of the haemolymph of the southwestern corn borer and the European corn borer in relation to their diapause. J Insect Physiol 29= 735–740.

Storey JM, Storey KB (1990) Carbon balance and energetics of cryoprotectants synthesis in a freeze-tolerant insect= responses to perturbation by anoxia. J Comp Physiol B 160= 77–84.

Goto M, Fujii M, Suzuki K, Sakai M (1997) Factors affecting carbohydrate and free amino acid content in overwintering larvae of Enosima leucotaeniella. J Insect Physiol 44= 87–94.

Goto M, Li YP, Kayaba S, Outani S, Suzuki K (2001) Cold hardiness in summer and winter diapause and post-diapause pupae of the cabbage armyworm, Mamestra brassicae L. under temperature acclimation. J Insect Physiol 47= 709–714

Storey KB, Churchill TA, Joanisse DR (1993) Freeze tolerance in hermit flower beetle (Osmoderma eremicola) larvae. J Insect Physiol 39= 737–742.

Okasaki T, Yamashita O (1981) Changes in glucose and fructose contents during embryonic development of the silkworm Bombyx mori. J Sericult Sci Jpn 50= 190– 196.

Osanai M, Yonezawa Y (1986) Changes in amino-acid pools in the silkworm Bombyx mori during embryonic life= alanine accumulation and its conversion to proline during diapause. Insect Biochem 16= 373–380.

Costanzo JP, Lee RE Jr, Lortz PH (1993) Physiological responses of freeze-tolerant and -intolerant frogs= clues to evolution of anuran freeze tolerance. Am J Physiol Regul Integr Comp Physiol 265= R721–R725.

Holmstrup M, Overgaard J, Bindesbøl A-M, Pertoldi C, Bayley M (2007)Adaptations to overwintering in the earthworm Dendrobaena octaedra= Geneticdifferences in glucose mobilisation and freeze tolerance. Soil Biol Biochem 39=2640–2650.

Calderon S, Holmstrup M, Westh P, Overgaard J (2009) Dual roles of glucose inthe freeze-tolerant earthworm Dendrobaena octaedra= cryoprotection and fuel for metabolism. J Exp Biol 212= 859–866.

Overgaard J, Malmendal A, Sørensen JG, Bundy JG, Loeschcke V, et al (2007)Metabolomic profiling of rapid cold hardening and cold shock in Drosophilamelanogaster. J Insect Physiol 53= 1218–1232.

MacMillan HA, Guglielmo CG, Sinclair BJ (2009) Membrane remodeling and glucose in Drosophila melanogaster= a test of rapid cold-hardening and chillingtolerance hypotheses. J Insect Physiol 55= 243–249.

Koštál V, Zahradnickova H, Simek P, Zeleny J (2007) Multiple component system of sugars and polyols in the overwintering spruce bark beetle, Ips typographus. J Insect Physiol 53= 580–586.

Soudi S, Moharramipour S (2012) Seasonal patterns of the thermal response in relation to sugar and polyol accumulation in overwintering adults of elm leaf beetle, Xanthogaleruca luteola (Coleoptera= Chrysomelidae). J Therm Biol 37= 384–391.

Kruger NJ, von Schaewen A (2003) The oxidative pentose phosphate pathway= structure and organisation. Curr Opin Plant Biol 6= 236–246.

Yaginuma T, Yamashita O (1979) NAD-dependent sorbitol dehydrogenase activity in relation to the termination of diapauses in eggs of Bombyx mori. Insect Biochemistry 9= 547–553.

Salvucci ME (2000) Sorbitol accumulation in whiteflies= evidence for a role in protecting proteins during heat stress. J Therm Biol 25= 353–361.

Wang X, Kang L (2005) Differences in egg thermotolerance between tropical and temperate populations of the migratory locust Locusta migratoria (Orthoptera=Acridiidae). J Insect Physiol 51= 1277–1285.

Hendrix DL, Salvucci ME (1998) Polyol metabolism in homopterans at high temperatures= accumulation of mannitol in aphids (Aphididae= Homoptera) and sorbitol in whiteflies (Aleyrodidae= Homoptera). Comp Biochem Physiol A 120= 487–494.

Koštál V, Slachta M, Simek P (2001) Cryoprotective role of polyols independent of the increase in supercooling capacity in diapausing adults of Pyrrhocoris apterus (Heteroptera= Insecta). Comp Biochem Physiol B 130= 365–374.

Saeidi F, Moharramipour S, Barzegar, M. Seasonal patterns of cold hardiness and cryoprotectant profiles in Brevicoryne brassicae (Hemiptera= Aphididae). Environ Entomol, In press.

Watanabe M, Tanaka K (1999) Seasonal change of the thermal response in relation to myo-inositol metabolism in adults of Aulacophora nigripennis (Coleoptera= Chrysomelidae). J Insect Physiol 45= 176–172.

Hodkova M, Hodek I (2004) Photoperiod, diapause and cold-hardiness. Eur J Entomol 101= 445–458.

Gekko K, Timasheff SE (1981) Mechanism of protein stabilization by glycerol= preferential hydration in glycerol-water mixtures. Biochemistry 20= 4667–4676.

Salt RW (1961) Principles of insect cold hardiness. Annu Rev Entomol 6= 55–74.

Storey KB, Storey JM (1988) Freeze tolerance in animals. Physiol Rev 68= 27–84.

Pullin AS, Bale JS (1989) Influence of diapause and temperature on cryoprotectant synthesis and cold hardiness in pupae of Pieris brassicae. Comp Biochem Physiol A 94= 499–503.

SUPLEMMENTARY DATA

Supplementary data 1 A picture of the GC-MS system., which consists of a Trace GC Ultra chromatograph, a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA), and CTC CombiPal autosampler (GERSTEL GmbH and Co.KG, Mülheim an der Ruhr, Germany) that automatized all the derivatization process.

Supplementary data 2 List of metabolites identified in females of *Tretranychus urticae* by GC/MS. Metabolites were classified according to eight categories= amino acids, polyols, sugars, intermediates of the citric acid cycle, other metabolites, non-quantified metabolites, internal standard and metabolites not found in any treatment. Non-quantified metabolites= the metabolites were detected but their concentrations were below the quantification limit (<QL) of the GC-MS. The minimal QL above which the metabolites can be accurately quantified is given in µM for each compound. Minimum and maximum concentrations (nmoles/mg fresh mass) detected among all treatments are also shown.

Metabolites	**Minimum and maximum metabolite concentrations (nmoles /mg fresh mass)**
Amino acids	
Alanine	0.17 - 0.20
Glutamate	4.11 - 23.66
Glycine	1.89 - 12.88
Isoleucine	0.25 - 4.88
Leucine	0.37 - 12.95
Ornithine	0.36 - 1.76
Phenylalanine	1.23 - 9.27
Proline	0.32 - 9.57
Serine	3.42 - 15.76
Threonine	0.69 - 8.02
Tryptophane	1.17 - 4.66
Valine	0.35 - 9.04
Polyols	
Adonitol	0.24 - 0.39
Arabitol	0.46 - 1.47
Galactitol	0.15 - 0.42
Glycerol	0.20 - 2.20
Glycerol 3 Phosphate	1.19 - 9.90
Inositol	0.45 - 1.88
Mannitol	0.14 - 4.95
Sorbitol	0.48 - 5.88
Xylitol	0.16 - 0.41
Sugars	
Fructose	0.72 - 5.42
Fructose 6 Phosphate	0.27 - 1.17
Galactose	0.15 - 0.42
Glucose	17.83 - 70.75
Glucose 6 Phosphate	0.29 - 0.67
Maltose	0.20 - 1.18
Mannose	0.48 - 1.94
Ribose	0.39 - 2.86
Intermediates of the citric acid cycle	
Citrate	0.94 - 8.65
Fumarate	2.55 - 5.15
Malate	1.46 - 14.58
Other Metabolites	
Cadaverine	0.02 - 0.25
Ethanolamine	0.58 - 2.65
Gluconolactone	0.16 - 6.66

Glycerate	0.15 - 0.52
Phosphoric Acid	0.81 - 4.31
Putrescine	0.25 - 1.39
Quinate	0.13 - 0.34
Triethanolamine	0.14 - 0.38
Non-quantified metabolites	
Dopamine	< QL (5 µM), S/N < 10
Erythritol	< QL (1 µM)
Galacturonic acid	< QL (5 µM), S/N < 10
Gentobiose	< QL
Saccharose	< QL (5 µM), S/N < 10
Succinate	< QL (20 µM)
Trehalose	< QL (5 µM), S/N < 10
Internal standard	
Arabinose	
Metabolites not found in any treatment	
Citrulline	NF
Glutamine	NF
Lactose	NF
Maltose	NF
Melezitose	NF
Melibiose	NF
Raffinose	NF
Spermine	NF
Urate	NF
Xylose	NF

Metabolic response to salt stress and diet in the ground-dwelling spider *Arctosa fulvolineata* (Araneae, Lycosidae) - (Article VII)

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Abstract

Soil salinity and the salinity of trophic resources may alter the osmoregulatory processes of arthropod, challenging the smooth regulation of body water, and, ultimately, survival. The intra and extracellular build-up of osmolytes represent a common strategy to attenuate acute hyperosmotic stress in several arthropod species. In the present study, we aimed to determine the impact of substrate and trophic resource salinities on salt tolerance in the female wolf spider, *Arctosa fulvolineata*, which is considered a specialist salt marsh species. We evaluated adult female survival and egg laying, and quantified the osmo-induced accumulation of compatible solutes (GC-MS). Three concentrations of substrate salinity were tested (0‰, 35‰ and 70‰) under three trophic conditions (starved spiders, spiders fed with salt prey (intertidal amphipods) and spiders fed with unsalted prey (freshwater amphipods)). We found no support for diet preferences in female *A. fulvolineata*, which exhibited similar predation rates on freshwater and marine amphipods. Survival and egg laying were significantly impaired when female *A. fulvolineata* were exposed to hypersaline conditions for 12 days. Our results showed an increase in the level of several compatible solutes when spiders were exposed for 12 days to saline conditions. For instance, α-alanine, β-alanine, arginine, asparagine, aspartate, homoserine, glutamine, glycine, proline and serine levels were 4–10 times higher under hypersaline conditions. The osmo-induced accumulation of amino acids may increase the osmolality of body fluids, thus enhancing the smooth regulation of body fluids and survival ability of wolf spider under extreme saline conditions.

Keywords= wolf spider, salinity, diet, amphipods, survival, egg laying, GC-MS, compatible solute, amino acid, alanine

1. INTRODUCTION

All living organisms must contend with fluctuating environmental parameters in their natural habitats (Clarke, 1991), including food and water availability, temperature, radiation and salinity (Hochachka & Somero, 2002). Soil salinity and the salinity of trophic resources may alter the osmoregulatory processes of organisms, challenging the smooth regulation of body water content (Verhoef & Witteveen, 1980; Elnitsky *et al.*, 2009). Arthropods exhibit two main strategies to maintain adequate osmotic pressure and cellular volume when exposed to saline conditions (Witteveen & Joosse, 1987; Charmantier & Charmantier-Daures, 1994). First, there are osmoconformers, which adjust body fluid osmolality to conform to the surrounding environment. Second, there are osmoregulators, which maintain a constant hemolymph and intracellular osmolality, regardless of habitat salinity.

In arthropods, salt is actively excreted *via* the Malpighian tubules, anal papillae and rectum or gills. Additional osmoregulation processes involve the osmo-induced accumulation of inorganic (Donini *et al.*, 2007) and organic compounds (Yancey, 2005). Organic compounds include polyols, sugars and free amino acids (Frolov *et al.*, 1991; McNamara *et al.*, 2004; Sowers *et al.*, 2006), which are synthesised by organisms from nutrients (Greenstone, 1979) or body stores. These osmolytes bind water molecules and increase hemolymph osmolality, thus limiting the amount of body water loss. In addition, these osmolytes balance the negative effects of NaCl on enzyme activities (Pollard & Wyn Jones, 1979). Thus, the intra and extracellular build-up of osmolytes represent a common strategy to attenuate acute hyperosmotic stress (Edwards, 1982). For example, in several species, proline, alanine and glycine are three amino acids that are associated with osmotic adjustments at the cellular level when environmental salinity increases (Larher *et al.*, 2003; Misra & Gupta, 2005). In addition, some organic compounds have protective effects on cellular proteins, and are termed 'chemical chaperones' (Welch & Brown, 1996).

Osmoregulatory processes have been well studied in marine vertebrates and invertebrates (Scudder *et al.*, 1972; Foster & Treherne, 1976; Witteveen *et al.*, 1987), but remain poorly investigated in strictly terrestrial species (Butt & Taylor, 1991). Salt marshes are interesting habitats for studying the salinity tolerance of non-marine arthropods, as they

host a large diversity and abundance of terrestrial taxa (mainly spiders, with an α-diversity of up to 100 species, and carabid beetles, Pétillon *et al.*, 2005a, 2008). These intertidal ecosystems are characterised by periodic immersion and emersion phases that result in alternating wet and dry cycles. These cycles, together with high salt concentrations, result in unique assemblages of specialist species, and decreasing species richness along salinity gradients (Pétillon *et al.*, 2008). Biological and physiological aspects of the salinity tolerance of salt-marsh arthropods have been subject to limited investigation (Cheng, 1976). For instance, Heydemann (1970) and Coleen *et al.* (1984) showed that osmoregulation is non-compulsory in a salt habitat for certain species of spider, with some obtaining water from the bodies of prey items (Vollmer & MacMahon, 1974; Butt & Taylor, 1986). In addition, the salt load caused by drinking water has been assumed to alter the composition of hemolymph in spiders (Coleen *et al.*, 1984); however, this assumption was based on biochemical comparisons between sea water and the hemolymph of spiders, without any supporting empirical data.

In this study, we investigated the physiological plasticity of the wolf spider, *Arctosa fulvolineata*, to saline conditions and trophic resources (i.e. prey from saline and freshwater habitats). This species is a dominant ground-dwelling predator in salt marshes (Pétillon *et al.*, 2005b). We hypothesised that (i) wolf spider females are characterised by a large salinity tolerance, and adjust feeding behaviour with respect to substrate salinity and (ii) changes in substrate salinity result in an osmo-induced accumulation of compatible solutes, thus enhancing the survival ability of wolf spider females exposed to saline conditions. We also evaluated adult female survival and egg laying ability to determine whether salinity tolerance is balanced by a trade-off with other life-history traits.

2. MATERIAL & METHODS

2.1. Species and sample sites

Wild adults of *A. fulvolineata* (Araneae, Lycosidae) were hand-collected at an intertidal salt marsh (Le Vivier-sur-Mer, 48° 36′08″N, 1° 46′27″W, France) in May 2009. This bay is characterised by a high tidal range (tidal amplitude potentially reaches 15 m) across a large

intertidal area (250 km² are submerged for 2–4 h once or twice a month). In total, 150 gravid females, which can be easily recognized by their large and distended opisthosoma, were sampled during the peak reproductive period for this species (Pétillon *et al.*, 2009a; Puzin *et al.*, 2011). The females were then kept for 1 day under controlled conditions, at a constant temperature of 20 °C (R.H. of 70 ± 5%, light/dark cycle 16h/8h) before being used for the experiments.

In the experiments, spiders were fed two amphipods species, *Orchestia gammarella* and *O. cavimana* (Amphipoda, Talitridae). *Orchestia gammarella* is a supra-littoral terrestrial species that is a known prey item of *A. fulvolineata* (Pétillon *et al.* 2009b), while *O. cavimana* inhabits the freshwater environments of estuaries (Morritt, 1988). A total of 500 *O. gammarella* individuals were collected from Le Vivier-sur-Mer, at the same time as the spiders. *Orchestia cavimana* individuals (N = 500) were collected at the end of April 2009, along the banks of the river Schelde (51° 3′0″N, 3° 43′0″W, Belgium), in an area that was 100 km far away from the coast to ensure that they were in a freshwater habitat. Both amphipod species were kept in plastic vials that were half-filled with water and substrate from the respective collection sites.

2.2. Chronic exposure to saline conditions

Salinity tolerance was examined by transferring wolf spiders into plastic vials (6 cm diameter, 7 cm depth) that were half-filled with 82 g of river sand saturated with water. Three salinity levels were used= (i) non saline (sand and minerals water without any additional salt, 0‰ salt); (ii) medium salinity (sand and mineral water with 35‰ salt); and (iii) high salinity (sand and mineral water with 70‰ salt). This final condition corresponds to the highest salinity level that wolf spiders experience in salt marshes, either at the lowest elevation above sea level or just after tidal floods (Pétillon *et al.*, 2010).

For each salinity condition, wolf spiders were randomly assigned to one of three experimental conditions= (i) food deprived (FD; N = 48); (ii) fed with *O. gammarella* (‘salted prey’= SP; N = 50); or (iii) fed with *O. cavimana* (‘unsalted prey’= USP; N = 50). The spiders were then kept in climatic chambers for 12 days at 20 °C (RH of 70 ± 5%,

light/dark cycle 16h/8h). The spiders in the latter two groups were fed every 4 days, so that each individual received three items over the course of the experiment. Wolf spider survival and egg laying were monitored every 2 days until the end of the experiment.

Metabolic fingerprinting was performed on whole body extracts of *A. fulvolineata* exposed for 12 days to the three saline conditions (0, 35 and 70‰), and to the three trophic conditions (unfed, salted prey, unsalted prey). Egg sacs were not included in the metabolic fingerprinting analysis. For each experimental condition, seven samples were prepared, each containing a pool of two spiders. The samples were directly ground in liquid nitrogen, lyophilized (dry mass), and stored at –20 °C until analysis. A total of 63 samples were prepared.

2.3. Metabolic fingerprints

All samples were weighed (dry mass), ground in 1000 µL of methanol-chloroform solution (2=1) and homogenised with 3 mm tungsten beads for 2 min at 30/s frequency (Retsch ™ MM301, bead-beating). A 500 µL volume of ultrapure water was added (final methanol-chloroform solution 2=1=2), and the samples were vortexed vigorously before centrifugation at 4,000g for 10 min at 4 °C. An 800 µL volume of the upper phase, which contained polar metabolites (including amino acids, polyols and sugars) was transferred to clean vials and vacuum-dried. A 500 µL volume of ultrapure water was added to the residuals, and the samples were stored at –20 °C before analysis.

2.3.1. Amino acids

A 20 µL volume of each of the samples was diluted in 60 µL of ultrapure water. Then, 5 µl of each sample was used to derive amino acids according to the AccQTag Ultra Derivatisation Kit protocol (Waters Corporation, Milford, MA). Amino acids were analysed using an Acquity UPLC® system (Waters Corporation, Milford, MA) by injecting 1 µl of the derivatisation mix onto an Acquity UPLC® BEH C18 1.7 µm 2.1 × 100 mm column heated at 55 °C, as described in Renault *et al.* (2010). Derivatised amino acids were

detected at 260 nm using a photo diode array detector. Peaks were identified according to the retention time compared with commercial standards.

2.3.2. Sugars and polyols

For GC-MS (Gas Chromatography-Mass Spectrometer) profiling of non-structural carbohydrates, polyols and organic acids, we used the method described by Renault *et al.* (2010). A 50 µL volume of each sample was vacuum-dried. Then, 50 µl of freshly prepared methoxyamine hydrochloride solution in pyridine (20 mg/ml) was added. Samples were agitated for 90 min at 30 °C before adding 50 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; Sigma, #394866). Samples were kept at 37 °C for 30 min, and then incubated at room temperature for 8 h. The GC-MS system consisted of a TriPlus autosampler, a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA). The injector temperature was maintained at 260 °C. The oven temperature ranged from 70 °C to 300 °C at a rate of 5 °C/min, and the oven was maintained at 300 °C for 5 min. Chromatograms were analysed using X-Calibur® software (Thermo Inc.). For each compound, standard solutions were run at 100, 200 and 300 µM. Then, metabolites in the samples were identified based on their retention time, two identifying ions and one confirmation ion. The metabolite levels of the samples were expressed in relative units, in reference to each external standard and dry mass of the samples.

2.4. Statistical analysis

Metabolite concentrations were first log-transformed ($x = \log_{10}(X + 1)$) to fulfil the assumption of normal distributed residuals. Metabolic differences among nine treatment levels were evaluated; specifically, *A. fulvolineata* exposed to 0‰ (FD, SP, USP), 35‰ (FD, SP, USP) and 70‰ (FD, SP, USP). As sampled spiders had a similar initial dry mass, the compounds were expressed as µmol/sample, to smooth the differential effect of egg laying among samples, which drastically decreased the dry mass of the spiders. A total of 32 compounds from sugars, polyols, amino acids were identified; therefore, multivariate

methods were first employed in the data analysis. Compounds beyond the detection limits were eliminated from the analysis. Compounds exceeding 80% correlation with other compounds were discarded, to avoid redundancy and an overestimate in differences among classes in multivariate analyses. Using log-transformed data, we then performed MANOVAs to address physiological differences among the nine classes of individuals exposed to controlled conditions. Class separation based on amino acid data was subsequently investigated in a Linear Discriminant Analysis (LDA). Statistical significance of LDAs was checked by using permutation tests (10,000 permutations) with *R* 2.13.1 statistical software (R Development Core Team 2008).

To statistically assess differences among the nine experimental conditions, general linear models (GLMs) were realised using all of the component values as separate dependent variables, with female dry mass being the covariate, and substrate salinity and feeding being fixed factors. If the interaction between fixed factors was not significant, a second GLM was used to test significant effects of separated fixed factors, separately. Bonferroni's corrections for multiple comparisons were applied. For each analysis, the level of statistical significance used was $\alpha = 0.05$. Results were expressed as mean ± S.E. GLMs were performed using the Statistica- 7 software.

3. RESULTS

3.1. Survival and laying rate

Survival remained very high when spiders were exposed to salinity levels ranging from 0 to 70‰, except for the spiders fed with salted prey and exposed to 70‰ salinity (**Table 1**). No significant difference was found among the different treatments ($\chi^2 = 0.99$, df = 4, P=0.911). Egg laying was strongly and significantly reduced (almost null) under hypersaline conditions (**Table 1**) ($\chi^2 = 83.05$, df = 2, P<0.001).

3.2. Amino-acids and sugars

All of the measured metabolites may contribute toward increasing the osmolality of body fluids. Therefore, we compared the total concentration of the 32 compounds among the

nine experimental conditions. Total concentration significantly differed among wolf spiders exposed to distinct saline conditions ($F_{2,58}$ = 42.88, P<0.001), and significantly increased when substrate salinity was 70‰ compared to females exposed to 0‰ salinity (**Table 2**). Wolf spiders exposed to 35‰ salinity were characterised by an intermediate total metabolite pool (**Table 2**).

The metabolic signatures of females significantly differed among the three saline conditions ($F_{2,26}$ = 1.64, P<0.001); however, the metabolic signatures did not differ according to diet ($F_{2,26}$ = 0.67, P>0.05; **Fig. 1**). The first axis of the LDA accounted for 65.9% of total inertia, with between-class inertia being 9.3 times higher than within-class inertia (**Fig. 1**). This axis was mainly constructed by variations in the amount of α-alanine, β-alanine, arginine, homoserine, glutamine and proline. The wolf spiders exposed to salinity levels from 0 to 70‰ were sorted according to increasing levels of these six metabolites. The second axis accounted for 21.3% of total inertia, with between-class inertia being 4.1 times higher than within-class inertia.

General linear models confirmed the LDA results, when testing for differences among saline conditions and the diet of spiders after the 12 day experiment. Even if a trend was observed for isoleucine, leucine and phenylalanine (with the mean values for starved spiders being lower compared to those of fed females), diet had no significant effect on the 32 measured components (**Tables 2 and 3**). Conversely, saline conditions induced several changes in metabolite levels. For instance, 18 out of 32 components significantly increased when substrate salinity rose (**Tables 2 and 3; Fig. 2**). The interaction between salinity and diet was not significant, indicating that salinity had a strong effect on the metabolic signature, regardless of diet.

Table 1 Survival and egg laying rates of female *Arctosa fulvolineata* held for 12 days at salinities of 0, 35 and 70‰.

	Substrate salinity: 0‰		Substrate salinity: 35 ‰		Substrate salinity: 70 ‰	
	Survival	Egg laying	Survival	Egg laying	Survival	Egg laying
Food deprived	100%	81.25%	100%	87.5%	100%	12.5%
Salted prey	100%	93.75%	100%	75%	62.5%	0%
Unsalted prey	100%	81.25%	93.75%	43.75%	81.25%	0%

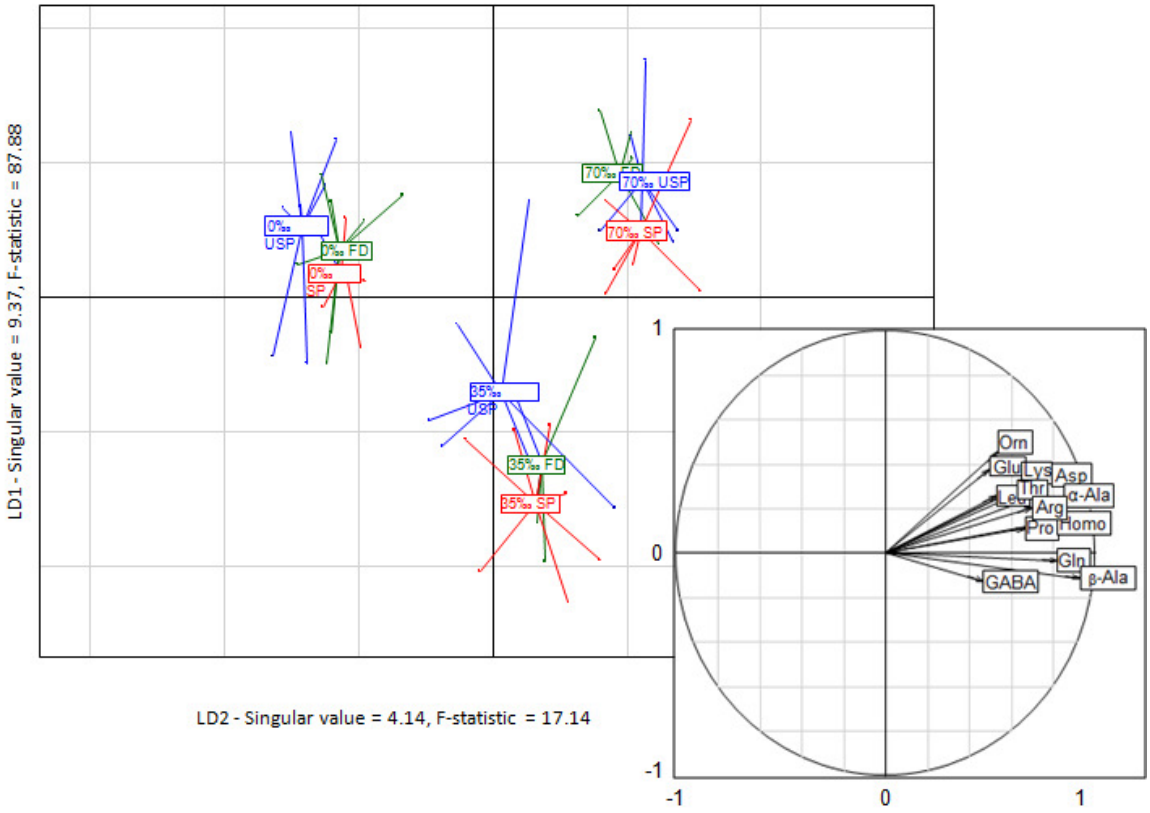

Fig. 1 Sample projection (63 samples) onto the first LDA discriminant plane in individuals maintained at zero salinity (0‰), medium salinity (35‰) and high salinity (70‰). The singular values represent the ratio of between- and within-class inertia. The lines link the samples (two individuals per sample) to the centroid of their class.
FD= food deprived spiders; SP= spiders fed with salted prey; USP= spiders fed with unsalted prey.
Amino-acids in the correlation circle are listed according to their official abbreviation. The correlations circle depicts the normed relation between each compound and the linear discriminant axes. Correlation varies from -1 to 1, with -1 and 1 meaning the highest contribution but in different directions and 0 means no contribution at all.

Table 2 Total amount of metabolites (means ± S.E.) in female *Arctosa fulvolineata* held at 0‰, 35‰ or 70‰ salinity at 20 °C for 12 days. Different letters denote significant differences among the experimental treatments (P<0.05).

	Total metabolite concentration (µmole/sample)		
	0‰	35‰	70‰
Starved	7.1 ± 2.0a	7.7 ± 3.1a	17.6 ± 4.1b
Unsalted prey	6.3 ± 1.2a	12.1 ± 3.9a,c	17.5 ± 6.3b,c
Salted prey	7.5 ± 3.1a	10.5 ± 3.9a,c	16.6 ± 1.9b,c

Table 3 Results of the GLMs for sugars and polyols. Substrate salinity and feeding regime were the fixed factors (each with three experimental treatments) and female dry mass was the covariate. X5 was an unidentified ose. Diet and the interaction between salinity and diet had no significant effect on the metabolite amounts (data not included in the table). For salinity, different successive letters indicate significant differences among the experimental treatments, which were compared with respect to 0‰ *versus* 35‰ *versus* 70‰.

	Salinity				**Dry Mass**				
Metabolites	Df	F	p	Post-hoc	Df	F	p	R^2 adj.	Df
Succinate	(4,50)	1.46	0.241		(2,54)	0.38	0.538		(1,54)
Malate	(4,50)	2.43	0.098		(2,54)	3.73	0.059		(1,54)
Glycoside_Galactopyranose	(4,50)	3.70	**<0.01**	a, b, ab (a<b)	(2,50)	0.66	0.421		(1,50)
Mannose	(4,50)	1.06	0.355		(2,50)	1.10	0.298		(1,50)
Glucose	(4,50)	1.94	0.154		(2,54)	0.63	0.431		(1,54)
Glucose_Galactose	(4,50)	0.91	0.408		(2,54)	16.87	**<0.001**	0.263 (+)	(1,54)
Myoinositol	(4,50)	1.21	0.306		(2,54)	11.07	**<0.001**	0.365 (+)	(1,54)
X5_Ose	(4,50)	2.06	0.137		(2,54)	0.27	0.603		(1,54)

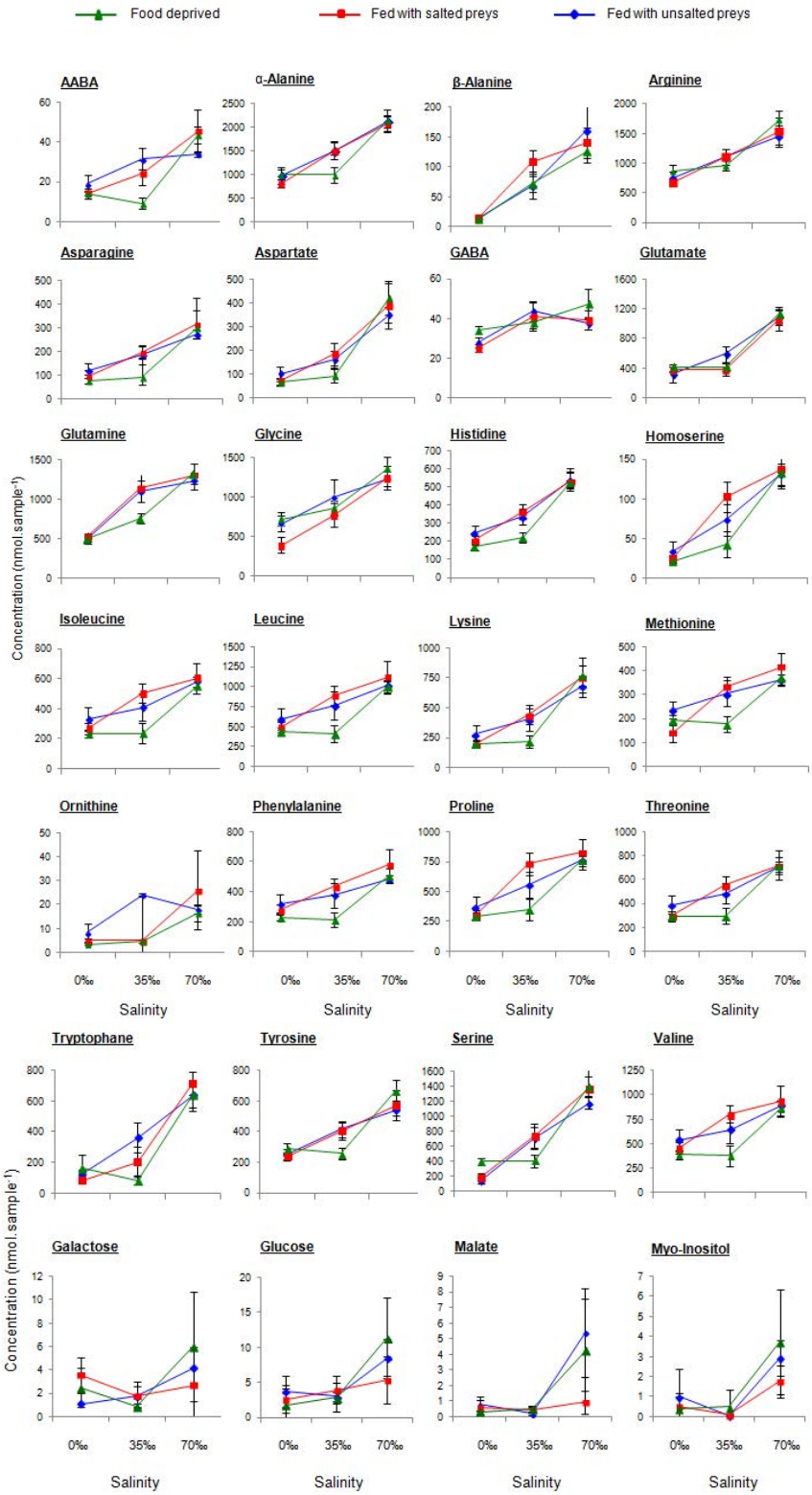
Food deprived
Fed with salted preys
Fed with unsalted preys
AABA
α-Alanine
β-Alanine
Arginine
Asparagine
Aspartate
GABA
Glutamate
Glutamine
Glycine
Histidine
Homoserine
Isoleucine
Leucine
Lysine
Methionine
Ornithine
Phenylalanine
Proline
Threonine
Tryptophane
Tyrosine
Serine
Valine
Galactose
Glucose
Malate
Myo-Inositol
Concentration (nmol.sample-1)
0‰
35‰
70‰
Salinity

Fig. 2 Variations in metabolite concentrations (means ± S.E.) of female *Arctosa fulvolineata* subject to 0‰, 35‰ or 70‰ salinity (blue). Green lines= food deprived wolf spiders; blue lines= wolf spiders fed with salted prey (SP); red lines= wolf spiders fed with unsalted prey (USP). Refer to Tables 3 and 4 for statistical results.

Table 4 Results of the GLMs for free amino-acids. Substrate salinity and feeding regime were the fixed factors (each with three experimental treatments) and female dry mass was the covariate. Diet and the interaction between salinity and diet had no significant effect on the metabolite amounts (data not included in the table). For salinity, different successive letters indicate significant differences among the experimental treatments, which were compared with respect to 0‰ versus 35‰ versus 70‰.

	Salinity				Dry mass				
Metabolites	Df	F	P	Post-hoc	Df	F	P	R^2 adj.	Df
AABA	(4, 51)	1.07	0.350		(2,55)	16.44	**<0.001**	0.486 (+)	(1,55)
α-Alanine	(4, 51)	9.54	**<0.001**	a<b<c	(2,55)	9.15	**<0.01**	0.506 (+)	(1,55)
β-Alanine	(4, 51)	11.89	**<0.001**	a<b<c	(2,55)	8.47	**<0.01**	0.431 (+)	(1,55)
Arginine	(4, 51)	7.07	**<0.001**	a<b<c	(2,55)	3.05	0.086		(1,55)
Asparagine	(4, 51)	5.90	**<0.01**	a<b<c	(2,55)	10.80	**<0.001**	0.502 (+)	(1,55)
Aspartate	(4, 51)	3.94	**<0.01**	a, a, b (a<b)	(2,55)	21.60	**<0.001**	0.615 (+)	(1,55)
GABA	(4, 51)	5.07	**<0.01**	a, b, b (a<b)	(2,55)	0.15	0.702		(1,55)
Glutamate	(4, 51)	20.90	**<0.001**	a, a, b (a<b)	(2,55)	0.16	0.688		(1,55)
Glutamine	(4, 51)	17.48	**<0.001**	a<b<c	(2,55)	2.37	0.130		(1,55)
Glycine	(4, 51)	10.49	**<0.001**	a<b<c	(2,55)	0.07	0.796		(1,55)
Histidine	(4, 51)	12.27	**<0.001**	a<b<c	(2,55)	6.18	**<0.01**	0.489 (+)	(1,55)
Homoserine	(4, 51)	7.72	**<0.001**	a<b<c	(2,55)	22.52	**<0.001**	0.574 (+)	(1,55)
Isoleucine	(4, 51)	1.58	0.216		(2,55)	17.39	**<0.001**	0.456 (+)	(1,55)
Leucine	(4, 51)	1.18	0.314		(2,55)	17.26	**<0.001**	0.449 (+)	(1,55)
Lysine	(4, 51)	3.66	**<0.05**	a, a, b (a<b)	(2,55)	18.00	**<0.001**	0.571 (+)	(1,55)
Methionine	(4, 51)	3.14	0.051		(2,55)	8.54	**<0.01**	0.369 (+)	(1,55)
Ornithine	(4, 51)	4.69	**<0.01**	ns	(2,55)	4.61	**<0.05**	-0.017	(1,55)
Phenylalanine	(4, 51)	0.67	0.516		(2,55)	18.21	**<0.001**	0.461 (+)	(1,55)
Proline	(4, 51)	5.18	**<0.01**	a<b<c	(2,55)	18.86	**<0.001**	0.484 (+)	(1,55)
Threonine	(4, 51)	2.63	0.081		(2,55)	14.67	**<0.001**	0.492 (+)	(1,55)
Tryptophane	(4, 51)	8.92	**<0.001**	a, a, b (a<b)	(2,55)	9.01	**<0.01**	0.533 (+)	(1,55)
Tyrosine	(4, 51)	3.52	**<0.05**	a<b<c	(2,55)	20.55	**<0.001**	0.579 (+)	(1,55)
Serine	(4, 51)	11.74	**<0.001**	a<b<c	(2,55)	17.13	**<0.001**	0.599 (+)	(1,55)
Valine	(4, 51)	1.24	0.299		(2,55)	16.42	**<0.001**	0.435 (+)	(1,55)

4. DISCUSSION

4.1. Contrasting effects of salinity on spider life history traits

Exposure to saline environments may have a negative effect on both the biological and physiological functions of organisms (Witteveen *et al.*, 1987; Ehlinger & Tankersley, 2004; Sowers *et al.*, 2006). Salinity may affect spiders when drinking interstitial saline water (Parry,

1954). In addition, hypersaline conditions may induce high osmotic stress on wolf-spiders, as demonstrated by an increase in the loss of body water when they are exposed to increased salinity levels (Pétillon *et al.*, 2011). Interestingly, we found that the survival rate of female *A. fulvolineata* remained very high, even when exposed to hypersalinemic conditions. These findings indicate that wolf spiders may be able to cope with saline conditions by excreting NaCl and/or by adjustments of internal osmolality due to the accumulation of compatible solutes. NaCl may be excreted *via* the coxal glands, as found in *Porrhothele antipodiana* (Mygalomorpha= Oipluridae) (Butt, 1983). The survival rate was lower when spiders were fed with salted prey and kept under hypersaline conditions. This observation indicates that the effects of salinity are cumulative, and that the resulting level of osmotic stress exceeded the range of salinity levels that this species is able to cope with.

Salinity had a strong deleterious impact on egg laying. Only three spiders laid egg sacs when were exposed to 70‰ salinity, regardless of diet. Conversely, the rate of egg laying at both non-saline and medium salinities under controlled conditions remained very high. These finding support the work of Witteveen & Joosse (1987), who also found that soil salinity had a negative effect on growth and egg laying rates in springtails (*Collembola* spp.).

The fitness of wolf spiders is generally improved by mono- and plurispecific food support (Greenstone, 1979), with the underlying body condition of spiders impacting subsequent egg production (Leborgne & Pasquet, 2005). Our results showed that the single-prey diet had no impact on subsequent egg laying, with both starved and fed females exposed to non- and medium saline conditions laying egg sacs, which contradicted the published literature (Greenstone, 1979; Leborgne & Pasquet, 2005). It is likely that longer periods of food deprivation might have been necessary to observe differences in egg laying between starved and fed spiders.

Although food specialisation in spiders is actually rare (mainly feeding on other predators, like ants or other spiders, Pekár & Toft, 2009; Toft *et al.*, 2010), some species are specialised to feed on decomposers (e.g. *Zodarion* species feed on woodlice, Řezáč & Pekár, 2007; Pekár *et al.*, 2010). Under natural conditions, adult *A. fulvolineata* may feed at high rates on amphipods, which constitute a very abundant food item in salt marshes (up to 1000

ind. m^{-2} for *O. gammarella*, Laffaille *et al.*, 2005). Our present study did not record a higher rate of predation on marine prey (salted preys) compared to freshwater prey (see Morrit, 1983; Morrit & Spicer, 1996 for a description of the hemolymph osmolality of these two amphipod species). Thus, it is unlikely that wolf spiders balance their body water regulation through water contained in the bodies of their prey items, as found in other species (Vollmer & MacMahon, 1974). Overall, our results indicate that prolonged exposure to hypersaline conditions impaired reproduction and survival of *A. fulvolineata*. However, the spiders were confined to small closed vials and had no access to freshwater; hence, we may have underestimated the physiological tolerance of this species to salinity under field conditions. For instance, the spider species *Amaurobioides africanus* requires a source of freshwater or diluted seawater to offset environment- and diet-induced salinity (Moloney & Nicolson, 1984).

4.2. Evident signs of the negative effects of salinity on spider physiology

Our statistical design maximised differences among experimental treatments, and sorted the experimental groups as a function of salinity level. Organic metabolites, such as amino acids and sugar alcohols, are widely known as important compounds in facilitating osmotic adjustment (Yancey, 2005). These compounds may have several osmoprotectant functions (Yancey, 2005), and are important for regulating hemolymph osmolality and prevent dehydration when specimens are exposed to hypersaline conditions (Nicolson, 1980; Benoit, 2010). In the present work, the quantity of several amino acids (α-alanine, β-alanine, arginine, asparagine, aspartate, homoserine, glutamine, glycine, proline and serine) was 4–10 times higher under hypersaline conditions. High amounts of β-alanine, glycine and serine have been previously observed in salt exposed crustaceans (Schoffeniels & Gilles, 1970). Glycine has been hypothesised to play an important cytoprotective role in mammalian cells (Zhang *et al.*, 2003), and has been reported to serve as a major osmolyte in invertebrates. High proline accumulation in intra- and extracellular compartments was found in the larvae of the mosquito *Culex tarsalis* (Diptera= Culicidae) to counterbalance the effects of hypersalinity stress (Patrick & Bradley, 2000a,b). Proline catches hydroxyl radicals induced by water deficits, and

has also been demonstrated to protect cytosolic and cellular enzyme structures during tissue dehydration (Sanchez *et al.*, 2008).

The large accumulation of alanine (α and β) is of particular note, which is a characteristic metabolic signature of all salt-exposed wolf-spiders, regardless of diet. Hyperalaninemia (increases up to 8 times under saline conditions) is a common response of salt-exposed animals, including several invertebrate taxa, such as insects (Patrick & Bradley, 2000a,b), springtails (Witteveen *et al.*, 1987) and polychaetes (Hoeger & Abe, 2004). This type of salt-induced bioaccumulation has also been recorded in plants (Garnett *et al.*, 2002).

The amassing of amino acids and other metabolites is often viewed and discussed in relation to their potential role as osmolytes and osmoprotectants (Yancey, 2005). However, in the present work, the level of nearly all amino acids increased under hypersaline conditions. However, some of these amino acids might not be involved in osmoregulatory processes. First, amino acids are important for egg synthesis in arthropods, as the proteins are used to construct the larval body. Hence, the accumulation of most amino acids under the highest saline conditions, together with impaired egg laying, might partially represent a secondary consequence of reduced oogenesis. Second, NaCl has detrimental effects on the activity of several enzymes (Pollard & Wyn Jones, 1979), and the bioaccumulation of a particular amino acid might also result from enzyme inactivation. For instance, Burton (1991) reported that high NaCl levels altered serine hydrolase activity, an enzyme involved in the serine catabolic pathway. Furthermore Patrick & Bradley (2000b) also recorded a significant osmo-induced rise of serine in *Culex quinquefasciatus* (Diptera= Culicidae); hence, it is possible that hypersaline conditions impair the activity of serine hydrolase, which, in turn, results in a rise of serine levels. A similar hypothesis might be drawn for aspartate, an amino acid that is not known to behave as a compatible solute, with aspartate transaminase activity being altered by hypersaline conditions.

Glucose concentrations remained similar among treatments, indicating that wolf spiders are able to fulfil their glucose requirements through neoglucogenesis. Glucose may be synthesised from numerous amino-acids and polyols. Besides, in the event of starvation, it has been shown that crustaceans used proteolyse to produce glucose. Lipids, like triglycerides, and amino-acids, like proline, have also been used as substrate to produce glucose.

5. CONCLUSION

In conclusion, our study highlighted that *A. fulvolineata*, which is a spider species endemic to salt marshes, thrives better under non-saline conditions; however, this species is able to withstand periods of immersion, lasting 2–4h, without any side-effects on other biological traits. Interestingly, our results did not support diet preferences in female *A. fulvolineata*. However, the significant effect of salinity on the metabolic signature of wolf spiders may have hidden diet-induced differences on the metabolic fingerprints. Under hypersaline conditions, survival and egg laying significantly declined when female *A. fulvolineata* were kept under hypersaline conditions, whereas the levels of most metabolites increased. Our results are globally consistent with the "compatible solutes strategy" theory (Oren, 1999), whereby large quantities of free amino acids amass that may enhance the smooth regulation of body water content.

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REFERENCES

Benoit, J.B., 2010. Water management by dormant insects= comparisons between dehydration resistance during summer aestivation and winter diapauses. Prog. Mol. Subcell. Biol. 49, 209-229.

Burton, R.S., 1991. Regulation of proline synthesis during osmotic stress in the copepod *Tigriopus californicus.* J. Exp. Zool. 259, 166–173.

Butt, A.G., Taylor, H.H., 1986. Salt and water balance in the spider, *Porrhothele antipodiana* (Mygalomorpha= Dipluridae)= Effects of feeding upon hydrated animals. J. Exp. Biol. 125, 85-106.

Butt, A.G., Taylor, H.H., 1991. The function of spider coxal organs= Effects of feeding and salt-loading on *Porrhothele antipodiana* (Mygalomorpha= Dipluridae). J. Exp. Biol. 158, 439-461.

Charmantier, G., Charmantier-Daures, M., 1994. Osmoregulation and salinity tolerance in zoeae and juveniles of the snow crab *Chionoecetes opilio*. Aquat. Living Resour. 8, 171-179.

Clarke, A., 1991. What is cold adaptation and how should we measure it. Amer. Zool. 31, 81-92.

Cheng, L., 1976. Marine insects. North Holland Publ. Co., Amsterdam.

Coleen, L., Moloney, C.L., Nicolson, S., 1984. Water relations and heamolymph composition of two intertidal Spiders (Order Araneae). Biology Ecology 83, 275-284.

Donini, A., Gaidhu, M.P., Strasberg, D.R., O'Donnell, M.J., 2007. Changing salinity induces alterations in hemolymph ion concentrations and Na^+ and Cl^- transport kinetics of the anal papillae in the larval mosquito, *Aedes aegypti*. J. Exp. Biol. 210, 983-992.

Edwards, H.A., 1982. Free amino acids as regulators of osmotic pressure in aquatic insect larvae. J. Exp. Biol. 101, 153-160.

Ehlinger G.S, Tankersley, R.A., 2004. Survival and development of horseshoe crab (*Limulus polyphemus*) embryos and larvae in hypersaline conditions. Biological Bulletin 206, 87-94.

Elnitsky, M.A., Benoit, J.B., Lopez-Martinez, G., Denlinger, D.L., Lee, R.E. Jr., 2009. Osmoregulation and salinity tolerance in the Antarctic midge, *Belgica antarctica*= seawater exposure confers enhanced tolerance to freezing and dehydration. J. Exp. Biol. 212, 2864-2871.

Foster, W.A., Treherne, J.E., 1976. Insects of marine saltmarshes= problems and adaptations. In= Cheng, L. (Eds.), Marine insects. North Holland Publishing Company, Amsterdam, pp. 5-42.

Frolov, A.V., Pankov S.L., Geradze K.N., Pankova S.A., 1991. Influence of salinity on the biochemical composition of the rotifer *Brachionus plicatilis* (Muller) aspects of adaptation. Comp. Biochem. Physiol. A 99, 541-550.

Garnett, M.R., Murch, S.J., KrishnaRaj, S., Dixon, M.A., Saxena, P.K., 2002. The rhizoflitration of sodium from hydroponic fluid using scented geraniums. Water, Air, & Soil Pollution, 140, 343-365.

Greenstone, M.H., 1979. Spider feeding behaviour optimises dietary essential amino acid composition. Nature 282, 501-503.

Hoeger, U., Abe H., 2004. □-Alanine and other free amino acids during salinity adaptation of the polychaete *Nereis japonica*. Comp. Biochem. Physiol. A 137, 161–171.

Heydemann, B., 1970. Ökologische Untersuchungen zum Problem der halophilen und haloresistenten Spinnen. Bulletin du Museum National d'Histoire Naturelle 41, 226-232.

Hochachka, P.W., Somero, G.N., 2002. Biochemical adaptation= mechanism and process in physiological evolution, Oxford University Press, Oxford.

Laffaille, P., Pétillon, J., Parlier, E., Valery, L., Ysnel, F., Radureau, A., Feunteun, E., Lefeuvre, J.C., 2005. Does the invasive plant *Elymus athericus* modify fish diet in tidal salt marshes? Estuar. Coast. Shelf Sci. 65, 739-746.

Larher, F.R., Aziz, A., Gibon, Y., Trotel-Aziz, P., Sulpice, R., Bouchereau, A., 2003. An assessment of the physiological properties of the so-called compatible solutes using in vitro experiments with leaf discs. Plant Physiol. Bioch 41, 657-666.

Leborgne, R., Pasquet, A., 2005. Time of oviposition and reproductive success in *Argiope bruennichi* (Araneae= Araneidae). Eur. J. Entomol. 102, 169-174.

McNamara, J.C, Rosa, J.C., Greene, L.J., Augusto, A., 2004. Free amino acid pools as effectors of osmostic adjustment in different tissues of the freshwater shrimp *Macrobrachium olfersii* (Crustacea, Decapoda) during long-term salinity acclimation. Mar. Freshw. Behav. Phy. 37, 193-208.

Misra, N., Gupta, A.K., 2005. Effect of salt stress on proline metabolism in two high yieldind genotypes of green gram. Plant Sci. 169, 331-339.

Moloney, C.L., Nicolson, S.W., 1984. Water relations and hemolymph composition of two intertidal spiders (Order Araneae). J. Exp. Mar. Biol. Ecol. 83, 275-284.

Morritt, D., 1988. Osmoregulation in littoral and terrestrial talitroidean amphipods (*Crustaceae*) from Britain. J. Exp. Mar. Biol. Ecol. 123, 77-94.

Morritt, D., Spicer, J.I., 1996. Developmental ecophysiology of the beachflea *Orchestia gammardus* (Pallas) (Crustacea= Amphipoda). I. Female control of the embryonic environment. J. Exp. Mar. Biol. Ecol. 207, 191-203

Oren, A., 1999. Bioenergetic aspects of halophilism. Microbiology and Biology Reviews 63, 334-348.

Nicolson, S.W., 1980. Water balance and osmoregulation in *Onymacris plana*, a tenebrionid beetle from the Namib Desert. J. Insect Physiol. 26, 315-320.

Parry, P.A., 1954. On the drinking of soil capillary water by spiders. J. Exp. Biol. 281, 218-227.

Patrick, M.L., Bradley, T.J., 2000a. Regulation of compatible solute accumulation in larvae of the mosquito *Culex tarsalis*= osmolarity versus salinity. J. Exp. Biol. 203, 831-839.

Patrick, M.L., Bradley, T.J., 2000b. The physiology of salinity tolerance in larvae of two species of *Culex* mosquitoes= the role of compatible solutes. J. Exp. Biol. 203, 821-830.

Pekár, S., Toft, S., 2009. Can ant-eating Zodarion spiders (Araneae= Zodariidae) develop on a diet optimal for euryphagous arthropod predators? Physiol. Entomol. 34, 195-201.

Pekár, S., Mayntz, D., Ribeiro, T., Herberstein, M. E., 2010. Specialist ant-eating spiders selectively feed on different body parts to balance nutrient intake. Animal Behaviour 79, 1301-1306.

Pétillon, J., Ysnel, F., Canard, A., Lefeuvre, J.C., 2005a. Impact of an invasive plant (*Elymus athericus*) on the conservation value of tidal salt marshes in western France and implications for management= Response of spider populations. Biol. Conserv. 126, 103-127.

Pétillon, J, Ysnel, F., Lefeuvre, J.C., Canard, A., 2005b. Are salt marsh invasions by the grass *Elymus athericus* a threat for two dominant halophilic wolf spiders? J. Arachnol. 33, 236-242.

Pétillon, J., Georges, A., Canard, A., Lefeuvre, J.C., Bakker, J.P., Ysnel, F., 2008. Influence of abiotic factors on spider and ground beetle communities in different salt marsh systems. Basic Appl. Ecol. 9, 743-751.

Pétillon, J., Puzin C., Acou, A., Outreman, Y., 2009a. Plant invasion phenomenon enhances reproduction performance in an endangered spider. Naturwissenschaften 96, 1241-1246.

Pétillon, J., Foucreau, N., Renault, D., 2009b. A propos de la consommation d'Amphipodes par l'espèce de marais salés *Arctosa fulvolineata* (*Araneae Lycosidae*). Nieuwsbrief van de Belgische Arachnologische Vereniging 24, 147-152.

Pétillon, J., Erfanzadeh, R., Garbutt, A., Hoffmann, M., 2010. Inundation frequency determines the successional pathway in a newly created salt marsh. Wetlands 30, 1097-1105.

Pétillon, J., Lambeets, K., Ract-Madoux, B., Vernon, P., Renault, D., 2011. Saline stress tolerance partly matches with habitat preference in ground-living wolf spiders. Physiol. Entomol. 36, 165-172.

Pollard, A., Wyn Jones, R.G., 1979. Enzyme activities in concentrated solutions of glycinebetaine and other solutes. Planta 144, 291-298.

Puzin, C., Acou, A., Bonte, D., Pétillon J., 2011. Comparison of reproductive traits between two salt-marsh wolf spiders (Araneae, Lycosidae) under different habitat suitability conditions. Anim. Biol. Leiden 61, 127-138.

R Development Core Team, 2008. R= A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, ISBN 3-900051-07-0, URL http=//www.R-project.org.

Renault, H., Roussel, V., Amrani, A.E., Arzel, M., Renault, D., Bouchereau, A., Deleu, C., 2010. The Arabidopsis pop2-1 mutant reveals the involvement of GABA transaminase (POP2) in salt stress tolerance. BMC Plant Biology 10 20. doi=10.1186/1471-2229-10-20.

Řezáč, M., Pekár, S., 2007. Evidence for woodlice-specialization in *Dysdera* spiders= behavioural versus developmental approaches. Physiol. Entomol. 32, 367-371.

Sanchez, D.H., Siahpoosh, M.R., Roessner, U., Udvardi, M., Kopka, J., 2008. Plant metabolomics reveals conserved and divergent metabolic responses to salinity. Physiol. Plantarum 132, 209-219.

Schoffeniels, E. Gilles, R., 1970. Osmoregulation in aquatic arthropods. In= Florkin, M., Sheer, B.T., (Eds), Chemical zoology. Vol. 5, Arthropoda. Academic Press, New York, pp. 255-286.

Scudder, G.G.E., Jarial, M.S., Choy, S.K., 1972. Osmotic and ionic balance in two species of *Cenocorixa*. J. Insect Physiol. 18, 883-895.

Sowers, A.D., Young, S.P., Grosell, M., Browdy, C.L., Tomasso, J.R., 2006. Hemolymph osmolality and cation concentrations in *Litopenaeus vannamei* during exposure to artificial sea salt or a mixed-ion solution= Relationship to potassium flux. Comp. Biochem. Physiol. A 145, 176-180.

Toft, S., Li, D.Q., Mayntz, D., 2010. A specialized araneophagic predator's short-term nutrient utilization depends on the macronutrient content of prey rather than on prey taxonomic affiliation. Physiol. Entomol. 35, 317-327.

Verhoef, H.A, Witteveen, J., 1980. Water balance in Collembola and its relation to habitat selection; cuticular water loss and water uptake. J. Insect Physiol. 26, 201-208.

Vollmer, A.T., MacMahon, J.A., 1974. Comparative Water Relations of five species of spiders from different habitats. Comp. Biochem. Physiol. A 47, 353-765.

Welch, W.J., Brown, C.R., 1996. Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperon 1, 109-115.

Witteveen, J., Joosse, E.N.G., 1987. Growth, reproduction and mortality in marine littoral Collembola at different salinities. Ecol. Entomol. 12, 459-469.

Witteveen, J., Verhoef, H.A., Letschert, J.P.W., 1987. Osmotic and ionic regulation in marine littoral Collembola. J. Insect Physiol. 33, 59-66.

Yancey, P.H., 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J. Exp. Biol. 208, 2819-2830.

Zhang, K., Weinberg, J.M., Venkatachalam, M.A., Dong, Z. 2003. Glycine protection of PC-12 cells against injury by ATP-depletion. Neurochem. Res. 28, 893–901.